ELIMINATION OF MICROPOLLUTANTS IN CONVENTIONAL AND NOVEL NITROGEN REMOVAL PROCESSES. A COMPARATIVE ASSESSMENT OF DIVERSE MICROBIAL COMMUNITIES CAPABILITIES

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Doctoral Thesis

Elimination of micropollutants in conventional and novel nitrogen removal processes - A comparative assessment of diverse microbial communities' capabilities

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CERTIFY THAT:

The doctoral thesis entitled: "**Elimination of micropollutants in conventional and novel nitrogen removal processes - A comparative assessment of diverse microbial communities' capabilities**" and presented by **Elissavet Kassotaki** to obtain a doctoral degree has been completed under our supervision and meets the requirements to opt for an ***International Doctorate***.

In witness whereof and for such purposes as may arise, the following certification is signed:

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Πάντα στον νου σου να ‘χεις την Ιθάκη.

Το φθάσιμον εκεί είν’ ο προορισμός σου.

Αλλά μη βιάζεις το ταξείδι διόλου.

Καλλίτερα χρόνια πολλά να διαρκέσει·

και γέρος πια ν’ αράξεις στο νησί,

πλούσιος με όσα κέρδισες στον δρόμο,

μη προσδοκώντας πλούτη να σε δώσει η Ιθάκη.

Η Ιθάκη σ’ έδωσε τ’ ωραίο ταξείδι.

Χωρίς αυτήν δεν θα ‘βγαινες στον δρόμο.

Άλλα δεν έχει να σε δώσει πια.

Κι αν πτωχική την βρεις, η Ιθάκη δεν σε γέλασε.

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Elissavet Kassotaki, Gianluigi Buttiglieri, Laura Ferrando-Climent, Ignasi Rodriguez-Roda, Maite Pijuan. 2016. Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products. Water Research: 94, 111-119.

Elissavet Kassotaki, Maite Pijuan, Adriano Joss, Carles M. Borrego, Ignasi Rodriguez-Roda, Gianluigi Buttiglieri. 2018. Unraveling the potential of a combined nitritation-anammox biomass towards the biodegradation of pharmaceutically active compounds. Science of the Total Environment: 624, 722–731.

Elissavet Kassotaki, Maite Pijuan, Ignasi Rodriguez-Roda, Gianluigi Buttiglieri. 2018. Comparative assessment of endocrine disrupting compounds removal in heterotrophic and enriched nitrifying biomass (submitted).

List of acronyms

ACE	**Ace**tate
AMO	**A**mmonia **Mo**nooxygenase
Anammox bacteria	**An**aerobic **Amm**onia **Ox**idizing (ANX) bacteria
Anammox process	**An**aerobic **Amm**onia **Ox**idation process
AOB	**A**mmonia **O**xidizing **B**acteria
ATU	**A**llyl**t**hio**u**rea
BNR	**B**iological **N**itrogen **R**emoval
BPA	**B**is**p**henol **A**
CBZ	**C**ar**b**ama**z**epine
COD	**C**hemical **O**xygen **D**emand
DO	**D**issolved **O**xygen
E1	**E**strone
E2	17β- **E**stradiol
E3	**E**striol
EDCs	**E**ndocrine **D**isrupting **C**ompound**s**
EE2	17α-**E**thinyl**e**stradiol
FISH	**F**luroscence ***I**n **S**itu* **H**ybridization
HET	**Het**erotrophic Bacteria
HRT	**H**ydraulic **R**etention **T**ime
IBU	**Ibu**profen
kbiol	Biodegradation rate constant
Kd	Solid-water partitioning coefficient
kH	Henry's law constant
MLSS	**M**ixed **L**iquor **S**uspended **S**olids
MLVSS	**M**ixed **L**iquor **V**olatile **S**uspended **S**olids
MTP	**Me**to**p**rolol
N	**N**itrogen
NAS	**N**itrifying **A**ctivated **S**ludge
NB	**N**itrifying **B**acteria
NH_4^+	Ammonium ion
NO_2^-	Nitrite

NO_3^-	Nitrate
NOB	**N**itrite **O**xidizing **B**acteria
PhACs	**Ph**armaceutically **A**ctive **C**ompound**s**
PNEC	**P**redicted **N**o-**E**ffect **C**oncentrations
N/A	Partial **N**itritation / **A**nammox
SAOR	**S**pecific **A**mmonium **O**xidation **R**ate
SBR	**S**equencing **B**atch **R**eactor
SBR-FN	**SBR** for **F**ull **N**itrification
SBR-PN	**SBR** for **P**artial **N**itrification
SD	**S**tandard **D**eviation
SFX	**S**ul**f**ametho**x**azole
SRT	**S**olids **R**etention **T**ime
TPs	**T**ransformation **P**roduct**s**
TSS	**T**otal **S**uspended **S**olids
VEN	**Ven**lafaxine
WWTPs	**W**aste**w**ater **T**reatment **P**lant**s**

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Summary

Micropollutants, such as pharmaceutically active compounds (PhACs) and endocrine disrupting compounds (EDCs), have emerged as a new threat and consequently a challenge for the scientific community as they can pose a significant risk to the environment and human health, undermining prosperity. The regulation of such compounds is however challenging and up-to-date a legal framework for drinking water, wastewater and water reuse has not yet been established. In fact, the diversity and continuous transformation of PhACs and EDCs in the human body and during sewage transport and treatment complicate their associated detection and the assessment of their toxicological significance. At the same time their release into the environment is neither particularly monitored nor controlled. One of the main points of concern that exacerbate the problem is the fact that wastewater treatment plants (WWTPs) cannot efficiently act as barriers to micropollutants' release and have been identified as main points of discharge and contamination of the aquatic environment. The fate of PhACs and EDCs as well as the extent of their removal in different engineered treatment systems are thus at the epicenter of current research. Nonetheless, up-to-date results on the efficiency of different technologies remain unclear and the necessity for more in depth exploration is being highlighted.

In this context, the present thesis aimed to determine the potential of both conventional and novel biological nitrogen removal processes in the simultaneous removal of nitrogen species, PhACs and EDCs and to evaluate the formation of main transformation products. The fate of five PhACs (ibuprofen, sulfamethoxazole, metoprolol, carbamazepine and venlafaxine) and five EDCs (estrone, 17β-estradiol, estriol, 17α-ethinylestradiol and bisphenol A) was investigated and a comparative assessment was carried out to determine the contribution of the autotrophic and heterotrophic bacteria present in different lab, pilot and full-scale treatment systems performing: nitrification, partial nitrification (i.e. nitritation), combined partial nitritation-anammox and aerobic oxidation of COD.

The experiments carried out identified critical factors triggering the elimination of the studied PhACs and EDCs and consequently the removal efficiency of the selected technologies. From one side, compound biodegradability was found to be a relatively accurate indicator of the total removal. Ibuprofen, 17β-estradiol and estriol -generally high biodegradable compounds- were greatly eliminated (up to 100%, 100% and 78%, respectively) under most

of the conditions applied. Contrariwise, carbamazepine, venlafaxine, 17α-ethinylestradiol and bisphenol A, that have been identified as persistent compounds, generally displayed low removals (< 20% in most cases) despite the diverse conditions, treatment technologies and types of biomass used. On the other hand, moderately biodegradable compounds (sulfamethoxazole, metoprolol and estrone) demonstrated a greater dependency on specific experimental factors such as redox conditions, nitrification rate as well as on unique biomass capabilities. Sulfamethoxazole removal, for example, demonstrated a great dependency on nitrification rate with higher removals being obtained at higher rates. For estrone, enhanced elimination was observed in all the cases that acetate was added in the system, whereas for metoprolol the highest degradation was displayed during anoxic conditions favoring anammox bacteria, results that highlight the capabilities of the heterotrophic and anammox fraction of the biomass, respectively. To be noted that experimental factors can be relevant also for the highly and poorly biodegradable compounds and led to deterioration or enhancement of their removal, respectively. In a point of fact, ibuprofen displayed a null removal under anoxic conditions aiming to heterotrophic denitrification, despite its high biodegradability. Different behavior was also observed in the case of the studied transformation products pointing out the necessity of a comprehensive monitoring of these by-products given their possible stability and toxicity.

Taken together, the results of the present thesis indicate that the fate of PhACs and EDCs cannot be easily predicted owing to the great variability within installations and operational conditions as well as the thousands of existing compounds. Nonetheless, the overall efficiency of wastewater treatment systems can be broadened by combining different aerobic and anaerobic conditions and different types of biomass.

Resumen

Los microcontaminantes, tales como los productos farmacéuticos (Pharmaceutical Compounds, PhACs) y compuestos de disrupción endocrina (Endocrine Disrupting Compunds, EDCs), se consideran una amenaza para el medio ambiente y la salud humana y en consecuencia su eliminación es una prioridad para la comunidad científica. La regulación para estos compuestos es difícil y hasta la fecha no existe un marco legal que controle o regule su presencia en aguas potables, residuales y/o de reúso. La diversidad y transformación continua de PhACs y EDCs en el cuerpo humano y durante el transporte y tratamiento de las aguas residuales complica su detección y la evaluación de su potencial toxicológico. Al mismo tiempo, su liberación en el medio ambiente no está particularmente estudiada ni controlada. Uno de los aspectos más problemáticos está relacionado con el hecho de que las depuradoras no pueden eliminar estos compuestos de manera eficiente y por lo tanto constituyen puntos principales de descarga en el medio acuático. Actualmente, el destino de los PhACs y los EDCs así como su grado de eliminación en distintos sistemas de tratamiento constituye el centro de muchas investigaciones. No obstante, la eficacia de distintas tecnologías aún no está clara, poniendo de manifiesto la necesidad de realizar estudios más exhaustivos.

En este contexto, la tesis presentada tiene como objetivo determinar el potencial de los procesos de eliminación biológica de nitrógeno (convencionales y los de nuevo desarrollo) en la eliminación simultánea de las especies de nitrógeno, de los PhACs y de los EDCs así como evaluar la formación de los principales productos de transformación de estos microcontaminantes. El destino de cinco PhACs (ibuprofeno, sulfametoxazol, metoprolol, carbamazepina y velafaxina) y cinco EDCs (estrona, 17β-estradiol, estriol, 17α-ethinylestradiol and bisphenol A) fue investigado. Se realizó una evaluación comparativa para determinar la contribución de las bacterias autótrofas y heterótrofas presentes en reactores a escala laboratorio, piloto y real donde se llevaba a cabo distintos procesos biológicos: nitrificación, nitrificación parcial (nitritación), combinación de nitritación parcial-anammox y oxidación aerobia de DQO.

Los experimentos llevados a cabo han identificado factores que potencían la eliminación de los PhACs y EDCs estudiados y en consecuencia la eficiencia de las tecnologías seleccionadas. Por un lado, se encontró que el potencial de biodegradación de un compuesto

es un indicador bastante preciso de su eliminación. El ibuprofeno, el 17β-estradiol y el estriol, generalmente considerados compuestos muy biodegradables, fueron mayoritariamente eliminados (hasta un 100%, 100% y 78% respectivamente) en las condiciones aplicadas. Por otro lado, carbamazepina, venlafaxina, 17α-ethinylestradiol and bisphenol A, que se han identificado como compuestos persistentes, generalmente presentaban eliminaciones bajas (<20% en la mayoría de los casos) a pesar de las distintas condiciones, tecnologías de tratamiento y tipos de biomasa utilizadas. Compuestos moderadamente biodegradables (sulfamethoxazol, metoprolol y estrona) han mostrado una gran dependencia de los factores experimentales aplicados como condiciones rédox, velocidad de nitrificación y características de la biomasa. La eliminación del sulfametoxazol, por ejemplo, mostró una gran dependencia con la velocidad de nitrificación, obteniéndose mayores eliminaciones a velocidades más altas. Para la estrona se observó una eliminación mejorada en todos los casos en que se agregó acetato en el sistema, mientras que para el metoprolol la degradación más alta se encontró en condiciones anóxicas, que favorecen las bacterias anammox, resultados que demuestran las capacidades de la fracción heterotrófica y anammox, respectivamente .Hay que destacar que los factores experimentales mencionados pueden ser relevantes también para los compuestos con mucha y poca biodegradabilidad y resultaron en la deterioración o mejora de su eliminación respectivamente. De hecho, el ibuprofeno mostró una eliminación nula en condiciones anóxicas, óptimas para la desnitrificación heterotrófica, a pesar de su alta biodegradabilidad. Distintos comportamientos se han observado en el caso de los compuestos de transformación estudiados, señalando la necesidad de una monitorización de estos sub-productos teniendo en cuenta su posible estabilidad y toxicidad.

En resumen, los resultados de la presente tesis indican que el destino de los PhACs y los EDCs no puede predecirse fácilmente debido a su gran variabilidad en las instalaciones y condiciones operacionales así como al gran número de los compuestos existentes. Aun así, la eficiencia de los sistemas de tratamiento de aguas residuales puede aumentarse, combinando condiciones aerobias y anaerobias y distintos tipos de biomasa.

Resum

Els microcontaminants, com els compostos farmacèutics (*Pharmaceutical Compounds*, PhACs) i els compostos de disrupció endocrina (*Endocrine Disrupting Compounds*, EDCs), han sorgit recentment com amenaça i repte per a la comunitat científica, ja que poden suposar un risc considerable per al medi ambient i la salut humana, perjudicant-ne la prosperitat. Tanmateix, la regulació d'aquests compostos és dificultosa i a dia d'avui encara no s'ha establert un marc legal que els reguli en aigües potables, residuals i/o reutilitzades. De fet, la diversitat dels PhACs i EDCs i la seva transformació contínua, tant en el cos humà com durant el transport i el tractament de les aigües residuals, compliquen la seva detecció i l'avaluació de la seva importància toxicològica. Al mateix temps, el seu alliberament en el medi ambient no està especialment supervisat i controlat. Un dels principals punts de preocupació que agreuja la problemàtica és el fet que les estacions depuradores d'aigües residuals (EDAR) no poden actuar de manera eficient com a barreres per a l'alliberament dels microcontaminants i s'han identificat com a punts principals de la seva descàrrega i contaminació del medi aquàtic. El destí dels PhACs i els EDCs, així com el seu grau d'eliminació en diferents sistemes de tractament, es troben, doncs, en l'epicentre de la investigació actual. No obstant això, els resultats obtinguts fins ara sobre l'eficiència de les diferents tecnologies encara no estan clars, destacant la necessitat d'una exploració més exhaustiva.

En aquest context, la present tesi pretén determinar el potencial tant dels processos convencionals com de nou processos biològics en l'eliminació simultània de les espècies de nitrogen, els PhAC i els EDC i avaluar la formació dels principals productes de transformació dels microcontaminants. S'ha investigat l'eliminació de cinc PhACs (ibuprofèn, sulfametoxazol, metoprolol, carbamazepina i venlafaxina) i cinc EDCs (estrona, 17β-estradiol, estriol, 17α-etinilestradiol i bisfenol A), realitzant una avaluació comparativa per determinar l'aportació de biomasses autotròfiques i heteròtrofes en sistemes de tractament diversos a escala laboratori, pilot i gran escala: nitrificació, nitrificació parcial (és a dir, nitritació), nitritació parcial combinada amb anammox i oxidació aeròbica de COD.

Els experiments dut a terme han identificat factors crítics que estan relacionats a l'eliminació dels PhACs i els EDCs estudiats i, en conseqüència, a l'eficàcia de tecnologies seleccionades. D'una banda, s'ha observat que la biodegradabilitat dels compostos pot ser un indicador

relativament precís de l'eliminació total. Ibuprofèn, 17β-estradiol i estriol - compostos generalment biodegradables – s'han eliminat molt (fins al 100%, 100% i 78%, respectivament) en la majoria de les condicions aplicades. D'altra banda, la carbamazepina, la venlafaxina, el 17α-etinilestradiol i el bisfenol A, que s'han identificat com a compostos persistents, generalment presenten baixes eliminacions (<20% en la majoria dels casos) malgrat les diverses condicions, tecnologies de tractament i tipus de biomassa utilitzats. D'altra banda, els compostos moderadament biodegradables (sulfametoxazol, metoprolol i estrona) han demostrat una major dependència de factors experimentals específics, com ara les condicions redox, la velocitat de nitrificació i les capacitats úniques de cada biomassa. L'eliminació del sulfametoxazol, per exemple, va ser altament dependent de la velocitat de nitrificació amb una major eliminació obtinguda a velocitats més altes. Per a l'estrona, es va observar una millor eliminació en els casos en què l'acetat era afegit al sistema, mentre que per al metoprolol es'observà una major degradació en condicions anòxiques, resultats que destaquen les capacitats de la fracció heterotròfica i anammox de la biomassa, respectivament. Cal assenyalar que els factors experimentals han estat rellevants també per als compostos altament i poc biodegradables ja que poden conduir al deteriorament o millora de la seva eliminació, respectivament, en alguns casos. De fet, l'eliminació de l' ibuprofèn va ser nul·la en condicions anòxiques, òptimes per a la desnitrificació heterotròfica, malgrat l'alta biodegradabilitat d'aquest fàrmac. També s'ha observat un comportament diferent en el cas dels productes de transformació estudiats, que apunta a la necessitat d'un seguiment integral d'aquests subproductes tenint en compte la seva possible estabilitat i toxicitat.

En conjunt, els resultats de la present tesi indiquen que el destí de PhACs i EDCs no es pot predir fàcilment a causa de la gran variabilitat dins de les instal·lacions i de les condicions operatives, així com dels milers de compostos existents. No obstant això, l'eficiència global dels sistemes de tractament d'aigües residuals es pot ampliar combinant diferents condicions aeròbiques i anaeròbies i diferents tipus de biomassa.

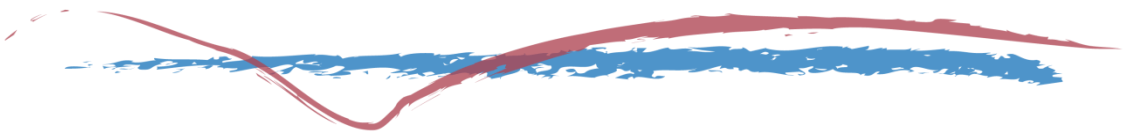
“Under the most rigorously controlled conditions of pressure, temperature, humidity and other variables, the organism will do as it damn well pleases”.

The Harvard Law

Block I

General Introduction, Objectives and Methodology

General Introduction



1.1 Micropollutants: the case of pharmaceutically active and endocrine disrupting compounds

Water is one of the most plentiful and essential compounds on planet earth. Today, however, water security is in jeopardy and water issues have been consistently listed as one of the top 10 threats to prosperity over the past 10 years (https://global.nature.org/content/water-for-life). One of the most challenging threats is the prevalence and continuous input of emerging contaminants, also known as micropollutants, into our water bodies.

The term micropollutants typically refers to organic compounds that are present at trace concentrations (ranging from a few ng/L to several μg/L) and their resistance to environmental degradation through chemical, biological, and photolytic processes, may have a negative effect on the environment and living organisms. They consist of a great variety of anthropogenic and natural substances such as pharmaceutically active compounds (PhACs), personal care products, endocrine disrupting compounds (EDCs), illicit drugs and other industrial chemicals such as pesticides, flame retardants and surfactants. Some of the micropollutants have only recently been developed and are newly introduced into the environment, whereas others have been prevalent for a long time but either have only recently been detected (due to the advances in analytical chemistry) or have only recently been recognized as potential hazards causing adverse effects on ecosystems or humans (Houtman, 2010). Currently in Europe there are approximately 3000 commercially available and commonly used pharmaceutical compounds (Ternes et al., 2015), while this number is expected to increase in the coming years as a consequence to the growing population, the improved quality of life and the high dependence of modern societies on chemicals.

1.1.1 Environmental impacts

The presence of micropollutants like PhACs and EDCs into the environment, has received great attention since the past two decades as, such compounds, are developed with the intention of having a beneficial biological effect on the organism to which they are administered but many of them often pass into the environment where they may exert unwanted biological effects (Halling-Sorensen et al., 1998). Nearly all categories of PhACs including pain killers (analgesics and anti-inflammatory), antibiotics (antibacterial),

antiepileptic drugs, beta-blockers, blood lipid regulators, x-ray contrast media, cytostatic drugs (chemotherapy) and oral contraceptives among others, have been found at concentrations up to the μg/L-level in sewage and surface samples (Heberer, 2002). These compounds are designed to be highly active and to interact with receptors in humans and animals, to be toxic for many infectious organisms and to be persistent so as not to become inactive before having a curing effect. At the same time, EDCs (either natural or synthetic) can interfere with the endocrine system by mimicking, blocking or also disrupting the function of hormones at certain doses (Bolong et al., 2009). It is thus because of the very nature and properties of both PhACs and EDCs that they may provoke unintended effects (such as bioaccumulation and biomagnification) on microorganisms and animals in the aquatic or terrestrial ecosystems especially towards species at top level of food chain (Boxall, 2004; Halling-Sorensen et al., 1998). Indeed, results from animal models, human clinical observations, and epidemiological studies converge to implicate EDCs as a significant concern to public health (Diamanti-Kandarakis et al., 2009), since they have even been accused for feminization of aquatic organisms which could give rise to reproductive and developmental abnormalities (De Gusseme et al., 2009; Forrez et al., 2008). The effects of EDCs toward animals are well reported. As regards to humans, direct effects are still debated but few studies suggest decrease in male sperm count, increase in testicular, prostate, ovarian and breast cancer and as a consequence significant implications toward fetuses and newborn babies due to reproductive malfunctions (Bolong et al., 2009). Similarly, PhACs exhibit most of the above mentioned harmful effects including inhibition of stimulation of growth, birth defects, and other developmental disorders and reproduction abnormalities (Bolong et al., 2009; Boxall, 2004; Kümmerer, 2009).

Thereupon, the presence of such compounds in the receiving water bodies (rivers, lakes and seas) raises concerns since they may even be used as sources for the abstraction of drinking water (Gros et al., 2012). Additionally, in the case of antibiotics there has been increasing evidence on the occurrence of both antibiotic resistant genes and antibiotic resistance bacteria in different environmental matrices such as water, sediments and biofilms (Marti et al., 2013; Subirats et al., 2017), that could have detrimental consequences. Finally, another point of concern is the fact that most of PhACs and EDCs still remain unregulated or are currently undergoing a regularization process, although directives and legal frameworks are not set-up yet (Rivera-Utrilla et al., 2013).

1.1.2 Legal framework in EU level and Switzerland

The diversity and relatively low concentrations of PhACs and EDCs complicate their associated detection and analysis procedures, while their toxicological significance is also difficult to assess. Regulation of these substances and their possible metabolites and transformation products (TPs) is hence challenging and commonly accepted limits for drinking water and wastewater have not yet been established for the global community. Even so, some legislative actions have been actively taken by the European Union.

A first step was made with the publication of the Water Framework Directive (2000/60/EC) that aimed to achieve good qualitative and quantitative status for all ground and surface waters (rivers, lakes, transitional waters, and coastal waters) in the EU. Moreover, a list of prioritized substances that are seen as a threat to surface and ground water was published (Directive 2013/39/EU) and member countries started monitoring and controlling their prevalence in September 2015. At the same time, in accordance with Article 16(2) of Directive 2000/60/EC, a watch list of substances for which Union-wide monitoring data are to be gathered for the purpose of supporting future prioritisation exercises was determined by Decision 2018/840 which repealed the previous implemented Decision 2015/495/EU. Fifteen substances have been included among which 3 EDCs: estrone, 17β-estradiol and 17α-ethinylestradiol and 5 PhACs belonging to the class of antibiotics: erythromycin, clarithromycin, azithromycin, amoxicillin and ciprofloxacin.

Switzerland is one of the first countries to implement national policy. The Swiss Water Protection Ordinance was revised and set into force in January 2016. It foresees the upgrade of 100 to 120 WWTPs, which represent about 50% to 60% of the municipal wastewater load in Switzerland, during the next 20 years (implementation by 2035) in order to remove at least the 80% of the inlet (after primary clarification) load of selected micropollutants. The selection of the WWTPs to be equipped with an advanced treatment (application of ozonation or powdered activated carbon and of a post-treatment) was based on different criteria as follows: a) WWTPs (> 80,000 inhabitants) with high loads, b) WWTPs (> 24,000 inhabitants) in the catchment of lakes, c) WWTPs (> 8,000 inhabitants) on rivers with a fraction of wastewater >10% and finally d) WWTPs (> 1,000 inhabitants) on rivers impacting drinking water resources. Twelve compounds (amisulpride, carbamazepine, citalopram, clarithromycin, diclofenac, hydrochlorothiazide, metoprolol, venlafaxine, benzotriazole, methyl-benzotriazole, candesartan, irbesartan) that: a) are identified as relevant and

representative for a larger group of micropollutants in Swiss surface waters, b) are not eliminated by biological treatment, c) are eliminated by both ozonation and powdered activated carbon to a similar extend and d) are detectable with a reliable and ready to use analytical method, have been selected as indicators of the removal efficiency (McArdell, 2016). Each canton has to choose 5 for monitoring through sampling campaigns (8-24 samples per year depending on the size of each WWTP). For each sampling the arithmetic mean of the individual removal efficiency of the 5 selected compounds has to be 80% (Hochstrat, 2015).

On the other hand, regarding new PhACs, the current European regulatory guidance requires new pharmaceuticals to undergo standard acute toxicity tests (to algae, *Daphnia magna* and fish) if the predicted or measured environmental concentration of the active ingredient is > 10 ng/L, according to the European threshold safety value, set by the European Medicines Agency (Gros et al., 2010).

1.1.3 Sources, occurrence and points of concern

Micropollutants make their way into the environment either through point sources such as the effluents of wastewater treatment plants (WWTPs), hospitals and various industries or through non point sources related to agricultural operations such as livestock (veterinary drugs, feed additives and manure), as well as to surface runoff of storm water or combined sewage overflow (CSO) (Figure 1.1).

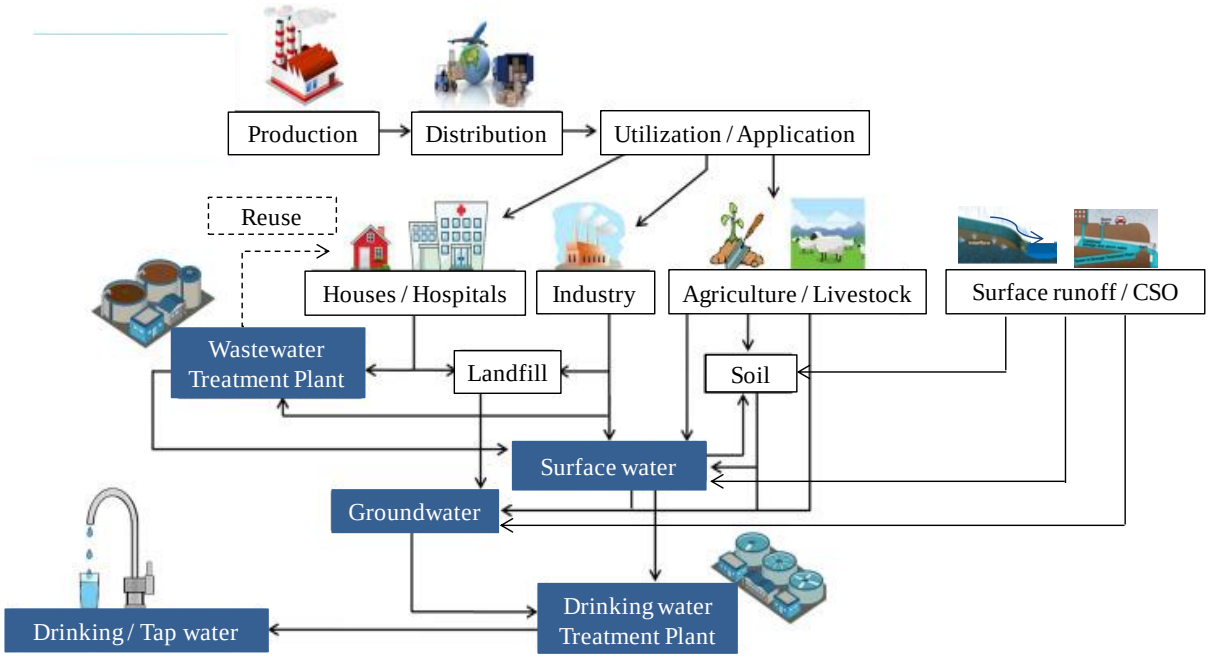


Figure 1.1: Representative sources and routes of micropollutants in the environment (adapted from Barbosa et al., 2016).

Their occurrence in wastewater as well as in surface, ground and drinking water, reveals significant spatial and temporal variations within the same or among different years and countries. The observed differences may rely on various factors such as production, administration and consumption rates; country practices, legislation and economic prosperity; degree of human metabolism (excretion rate); size, operational conditions and efficacy of WWTPs; climatic conditions such as temperature and rainfall (that affect the elimination efficacy and dilution factor of WWTP discharge) and finally environmental persistence, to name a few (Baker and Kasprzyk-Hordern, 2013; Göbel et al., 2005; Jelic et al., 2011; Kümmerer, 2009; Luo et al., 2014). Dilution factors in receiving surface waters themselves, hydrological characteristics of the water body and environmental removal processes are also critical parameters to be considered (Bonvin, 2013). Additionally, the easy accessibility and hence massive use of some compounds (e.g. ibuprofen) can lead to particularly high levels of occurrence (up to some µg/L), whereas local common diseases can also induce a higher consumption of specific PhACs during certain periods (Luo et al., 2014).

Accordingly, the presence, frequency of occurrence and origin of contamination of micropollutants are not always well understood. In addition, the actual environmental concentrations may be higher than the predicted no-effect concentrations (PNEC) (Al Aukidy

et al., 2014). Thereupon, although micropollutants are commonly found in waters at trace concentrations, their presence is still alarming for Water Utilities and Health Protection Agencies.

1.1.4 Fate and transformation of PhACs and EDCs

PhACs and EDCs that are consumed and/or produced by humans and animals, end up in sewage and WWTPs through either direct (in sewers, septic tanks, and trash) or indirect (excretion via urine and feces, surface runoff) disposal. Since the body can only metabolize a fraction of the administered compound, it may enter as an unchanged parent compound or in the form of metabolites and conjugates of glucuronic or sulphuric acid (Gros et al., 2010), which are the principal excreted metabolites for organic compounds by humans (Hamid and Eskicioglu, 2012; Ternes et al., 1999). The excretion rate is strictly correlated to each compound and to individual human characteristics including age, gender, health status and concurrent assumption of other substances (Al Aukidy et al., 2014). Metabolites and TPs can undergo further modification due to biological, chemical and physical processes (Jelic et al., 2015; Verlicchi et al., 2012) or even be cleaved back to the parent compound (Göbel et al., 2005; Hamid and Eskicioglu, 2012; Radjenović et al., 2007) during sewage transport and treatment, and in the aquatic systems. However, many of these substances are able to pass through wastewater treatment processes by virtue of their persistency or/and the continuous introduction (Luo et al., 2014), constituting WWTPs -that are not specifically designed to remove them- a main point of discharge into the environment (Buttiglieri and Knepper, 2008). Another point of concern is that exposure to complex mixtures as a result of the presence of metabolites (transformation in the human body) and TPs (transformation in the environment) could be more alarming owing to synergistic effects. Indeed, the toxicity of a mixture of non-steroidal anti-inflammatory drugs against daphnia was found to be considerable even at concentrations in which the single substances showed no or only very slight effects (Cleuvers, 2004). At the same time, the potential stability or toxicity of these byproducts is another issue to be considered. Some of them are more bioactive than their metabolic precursor (Rivera-Utrilla et al., 2013) whereas others, although less toxic in the long-term, might still have a high toxicity to living organisms within 48 h (Boxall et al., 2003). Environmental risk assessment and ecotoxicological studies for mixtures of such compounds are, however, very limited (Rivera-Utrilla et al., 2013) since it is generally difficult to estimate if adverse effects to non target organisms will occur at environmental

levels (Gros et al., 2010). Hence, the necessity of addressing the elimination or at least the fate of these byproducts during wastewater treatment is being underscored.

1.1.5 Mitigation strategies

The evidence of the adverse effects that PhACs and EDCs could pose on the environment and human health, coupled with the fact that the entire life cycle of a compound has to be taken into account to identify opportunities for risk management and risk reduction (Kümmerer, 2009), calls for immediate action and precautionary measures are being taken to reduce their release into the environment.

Up-to-date, the removal of these substances has mainly been relying on centralized end-of pipe solutions which are neither affordable in all countries nor that sustainable and should be avoided (Joss et al., 2006; Kümmerer, 2009). Various alternative approaches could thus be adopted in the long run and are already under investigation. These include segregation of sources (urine and feces separation, separate treatment of hospital wastewater), improvement of drug disposal information and waste containers, more considerate prescription of PhACs and EDCs by doctors, correct usage by patients, as well as promotion of green-pharmaceutical industry (Bonvin, 2013; Boxall, 2004; Joss et al., 2006). However, the promotion of green chemistry and the prohibition of toxic contaminants might be an ethical dilemma as when it comes to PhACs both humans and animals may rely on them for their survival. At the same time, although a combination of different strategies would be more effective, the delay in imposing the above mentioned measures is another critical factor to be considered. Consequently, optimization of the current wastewater treatment methods is of high importance and may represent an efficient and timely alternative in increasing the overall removal of these substances.

On the other hand, the increasing energy demand due to population growth and high living standards in combination with the need to maintain a reliable effluent quality raises concerns to many authorities. As a result, although the main aim of WWTPs is to reduce harmful emissions into water bodies, more attention is recently paid to energy efficiency and even broader to overall environmental sustainability (Schaubroeck et al., 2015). We thus need to opt for technologies that could integrate across different aspects, such as addressing micropollutants-related water quality issues at the lowest energy and carbon footprint possible.

1.1.6 Transformation mechanisms and pathways in WWTPs

The fate of micropollutants during wastewater treatment is determined by their physicochemical properties such as solubility, volatility, adsorbability, absorbability, biodegradability, and polarity as well as by wastewater treatment-related factors. Properties and operational parameters could vary greatly among compounds and WWTPs respectively, with obvious repercussions on the behavior and consequently on the removal efficiencies of micropollutants.

Commonly, the overall removal of micropollutants during these processes refers to the losses of a parent compound produced by different mechanisms of chemical and physical transformation (Jelic et al., 2011), including volatilization, sorption and biodegradation. In general, for many PhACs and EDCs, biodegradation / biotransformation and sorption are the two major removal mechanisms during biological treatment, while volatilization occurs to a minor degree (Verlicchi et al., 2012).

Volatilization is related to the transfer of micropollutants from the liquid to the gas phase and it normally occurs at the surface of a biological reactor when aeration is applied (stripping). It depends on the volatility of each compound which is expressed by the Henry's law constant (kH) and on operational parameters (aeration, agitation, temperature and atmospheric pressure). kH ranging from 10^{-2} to 10^{-3} (atm·m^3)/mol commonly indicates high tendency of volatilization (Stenstrom et al., 1989). According to literature, volatilization is totally negligible for PhACs and EDCs (Schröder et al., 2016; Suárez et al., 2008; Ting and Praveena, 2017), hence of minor importance in their removal.

Sorption is related to the transfer of micropollutants from the liquid to the solid phase and can be an important removal mechanism especially for poorly biodegradable compounds. It is being governed by many factors including pH, redox potential, stereo chemical structure and chemical nature of both the sorbent and the sorbed molecule (Kümmerer, 2009). The two main sorption mechanisms are: absorption (hydrophobic interactions characterized by the K_{ow} value, relevant for neutral compounds) and adsorption (electrostatic interactions related to the substance tendency to be ionized or dissociated in aqueous phase, characterized by the dissociation constant, pKa) (Schröder et al., 2016). In the first case hydrophobic pollutants interact with suspended solids, extracellular polymeric substances or the lipophilic cell membrane of microorganisms (Margot et al., 2015), whereas in the second positively charged groups of chemicals interact with the negatively charged surfaces of the microorganisms

(Ternes et al., 2004). The solid-water partitioning coefficient (Kd) has been proposed as a relative accurate indicator (Joss et al., 2005; Ternes et al., 2004) since it takes into account both absorption (K_{ow}) and adsorption (pKa). For compounds having Kd lower than 0.3 L/gSS (log Kd < 2.48), the sorption onto the secondary sludge is not relevant (Joss et al., 2005).

Biodegradation is related to a sequence of changes (breakdown) of the parent compound by the microorganisms present in activated sludge that could even lead to mineralization. It can occur via different mechanisms: a) single substrate growth of a small subset of specialist oligotrophic organisms, which is less common in WWTPs; b) co-metabolism, in which micropollutants are decomposed by enzymes generated for other primary substrate degradation (e.g. ammonia monooxygenase-AMO) and are not used as carbon and energy source for microbial growth; and c) mixed substrate growth, in which micropollutants are used as carbon and energy source and, eventually, become mineralized (Luo et al., 2014). In general, compounds could be classified according to their biodegradation rate constant (kbiol) into very highly (kbiol > 5 L/gSS d), highly (1< kbiol <5 L/gSS d), moderately (0.5< kbiol <1 L/gSS d) and hardly (kbiol <0.5 L/gSS d) biodegradable (Suarez et al., 2010).

Each of the aforementioned mechanisms could take place during the different stages of wastewater treatment. WWTPs generally consist of a preliminary treatment and two main stages; a primary and a secondary treatment. An optional tertiary treatment could be applied depending on the sensitivity of the receiving ecosystem or on the type of water reclamation but limitations are posed due to maintenance and operational costs.

The possible removal of micropollutants during the first two stages of treatment is explained below.

Primary treatment processes are used for the separation of the suspended (solids, fats, grease and oil) and liquid fraction of wastewater by aeration and sedimentation. The settled and floating materials are removed and the remaining liquid is then subjected to secondary treatment. Here, micropollutants could be removed by volatilization or by sorption on the primary sludge upon their kH and Kd coefficients.

Secondary treatment processes are used for the removal of dissolved and suspended biological matter by microorganisms. In this stage micropollutants are subjected to a range of processes, including dispersion, dilution, partition, sorption, biodegradation and abiotic transformation (Luo et al., 2014). The fraction of a pollutant removed by biodegradation

depends on operating parameters including the hydraulic retention time (HRT), the solids retention time (SRT) and redox conditions, as well as on wastewater characteristics such as pH and temperature. Both sludge concentration and composition which are determined by the SRT play a crucial role in activated sludge systems for nutrient removal. Under long SRTs, higher biodegradation efficiencies of different micropollutants have been reported, probably associated to the development of a more diverse microbial community, including the growth of nitrifying bacteria (Clara et al., 2005; Sathyamoorthy et al., 2013). A long HRT, which is the amount of time that allows for biodegradation and sorption, can also enhance the removal since micropollutants having slow/intermediate kinetics will experience less effective biodegradation at shorter HRTs or increasing loading rates (Fernandez-Fontaina et al., 2012). The biodegradation rate can also be influenced by temperature (e.g. higher degradation rate at 20°C compared with 10°C), pH (influences the enzymatic activity and cell uptake), redox conditions (usually higher under aerobic conditions), and the availability of a co-substrate (Margot et al., 2015).

It is hence deduced that many factors are triggering the removal of micropollutants and consequently the efficiency of a WWTP. Although data are scarcer, it seems that the primary treatment with or without enhancement has a limited effect on micropollutants (Carballa et al., 2017), and in some cases parent compounds may even be released during the process (i.e. higher concentration at the outlet when compared to the inlet) mainly as a result of deconjugation processes (Göbel et al., 2005). On the other hand, although enhanced tertiary treatment processes such as ozonation, powdered activated carbon dosing and chlorine dioxide oxidation, in combination with a biologically active downstream sand filtration, can be robust engineering solutions to eliminate the residual micropollutants deriving from the biological step (Plósz et al., 2010), very few WWTPs are currently equipped with these technologies specifically for micropollutants' removal (quaternary treatment). Therefore, the secondary treatment stage has a major effect on their mitigation before entering into the receiving environments (Men et al., 2017) and an ultimate effort on the optimization of the existing -most commonly used- activated sludge technologies is crucial.

1.2 Activated sludge processes for the removal of conventional pollutants

1.2.1 Conventional activated sludge processes

The inception of the activated sludge process was based on the need of removing dissolved and particulate organic matter from sewage. Since it has proven itself to be effective, versatile and durable, a number of different processes and reactors configurations have evolved since its early conception. A conventional activated sludge (CAS) system is usually designed to remove or to decrease the loads of the bulk organic and inorganic constituent that may otherwise pollute the receiving waters and lead to eutrophication (Buttiglieri and Knepper, 2008). However, with greater frequency, the CAS processes used today may incorporate nitrification, biological nitrogen removal (BNR) and/or biological phosphorus removal (Metcalf & Eddy, 2003). Several groups of heterotrophs and autotrophs can now be sustained together to perform the different functions required by these processes, and their metabolic activities may be predicted and individually controlled to ensure optimum process performance (Orhon, 2014).

1.2.2 Conventional and novel biological nitrogen removal processes

Nitrogen (N) removal from municipal and industrial wastewaters via the conventional nitrification-denitrification route has become a key stage in biological treatment over the past decades (Lackner et al., 2014). During nitrification, ammonium (NH_4^+) is first oxidized to nitrite (NO_2^-) by ammonia oxidizing bacteria (AOB) in a process called nitritation (Eq. 1.1) and then to nitrate (NO_3^-) by nitrite oxidizing bacteria (NOB) in a process called nitratation (Eq. 1.2).

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + 2\,H_2O \qquad \text{(Eq. 1.1)}$$

$$NO_2^- + 0.5\,O_2 \rightarrow NO_3^- \qquad \text{(Eq. 1.2)}$$

Nitritation is actually a two step process that is being carried out by different enzymes of AOB. The first step of oxidation (NH_4^+ to hydroxylamine) is carried out by AMO, whereas the second (hydroxylamine to NO_2^-) by Hydroxylamine oxidoreductase. On the other hand, during nitratation, NOB utilize the enzyme NO_2^- oxidoreductase to conduct the process.

Denitrification generally proceeds through four steps of reactions where different enzymes are involved as catalysts. First, NO_3^- is being reduced to NO_2^- (NO_3^- reductase), then to nitric

oxide (NO_2^- reductase), then to nitrous oxide (nitric oxide reductase) and finally to dinitrogen gas (N_2, nitrous oxide reductase). The complete process can be expressed as a net balanced redox reaction, where NO_3^- gets fully reduced to N_2 (Eq. 1.3).

$$2\,NO_3^- + 10e^- + 12\,H^+ \rightarrow N_2 + 6\,H_2O \qquad \text{(Eq. 1.3)}$$

This traditional nitrification-denitrification pathway is, however, energy intensive whereas addition of external carbon source is also required (for denitrification).

Currently, conventional systems are still widely employed for wastewater treatment mostly because they produce effluents that meet the required quality standards in terms of organic matter and nutrients removal (suitable for disposal or recycling purposes), at reasonable operating and maintenance costs (Jelic et al., 2011). However, these installations are facing a number of challenges mostly deriving from the need of reducing their footprint both physically (less land area required) and environmentally (less energy consumption, less sludge production, fewer pollutants and greenhouse gasses emissions, etc.) (Alvarino et al., 2018).

To tackle these challenges, research is directed towards more sustainable systems, and numerous novel BNR processes have been developed in the past several years, such as the partial nitritation, where NH_4^+ is oxidized to NO_2^- without generation of NO_3^-. This process is required for the subsequent application of anaerobic ammonium oxidation (anammox), where NH_4^+ as electron donor and NO_2^- as electron acceptor are converted anaerobically mainly into dinitrogen gas and some NO_3^- by anammox bacteria (Strous et al., 1998) (Eq. 1.4).

$$NH_4^+ + 1.32\,NO_2^- + 0.066\,HCO_3^- \rightarrow 1.02N_2 + 0.26\,NO_3^- + 0.66\,CH_2O_{0.5}N_{0.15} + 2.03\,H_2O \qquad \text{(Eq. 1.4)}$$

A schematic representation of the conventional and non conventional process is given below (Figure 1.2).

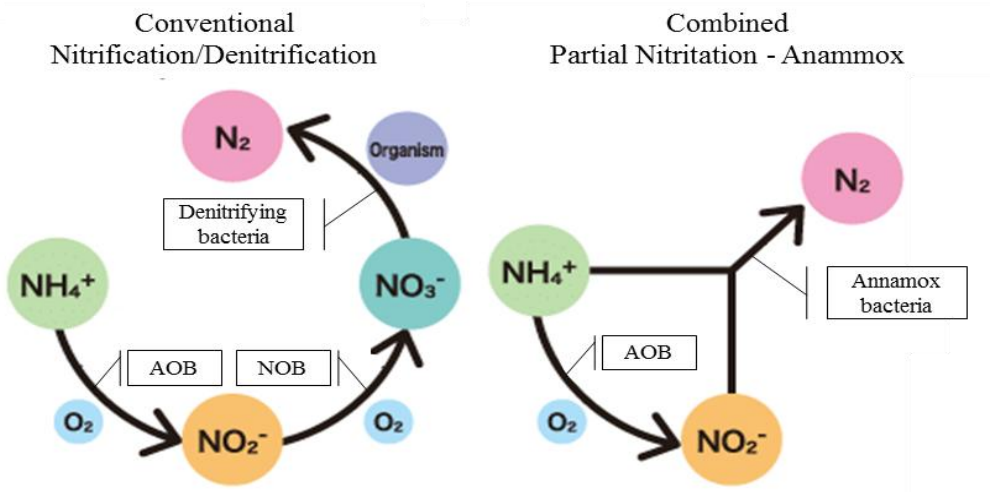


Figure 1.2: Schematic representation of nitrification/denitrification and combined partial nitritation-anammox processes (adapted from Aguirre-Sierra, 2017).

Nowadays, the combined partial nitritation-anammox (N/A) is the most promising alternative for nitrogen removal in WWTPs since it entails significant advantages over the conventional nitrification-denitrification pathway. It offers up to 60% reduction in aeration requirements and consequently in energy consumption, 100% savings in organic carbon (autotrophic bacteria) and up to 90% reduction in sludge production (Lackner et al., 2014; Laureni et al., 2015; Schaubroeck et al., 2015; van Loosdrecht and Brdjanovic, 2014), leading at the same time to a significant reduction in operational costs. Moreover, in combination with anaerobic digestion, the energy balance of wastewater treatment could turn out neutral or even positive (Siegrist et al., 2008).

Up-to-date, numerous processes have been successfully applied under mesophilic conditions and with different configurations (i.e. both processes of partial nitritation and anammox occur simultaneously or in separate reactors), bacteria growth types (granular sludge, activated sludge, biofilm) and reactor types. However, so far, these installations have been used for the treatment of high strength NH_4^+ wastewater (typically the reject water from the anaerobic digester - sidestream operation), which represents a small percentage of the total N loading in the WWTP. Recently though, despite the challenges of mainstream application, the potential and reliability of such systems in meeting the nitrogen discharge limits during the direct treatment of municipal wastewater under psychrophilic conditions has been confirmed (Laureni et al., 2016; Reino et al., 2018). Consequently, anammox based processes could

deliver sustainable environmental solutions working at the lowest energy and carbon footprint possible.

1.3 Conventional and novel BNR processes for the removal of micropollutants

The fate of PhACs and EDCs has been extensively studied in CAS systems as they represent the most commonly applied technology for wastewater treatment. Some of these compounds are susceptible to conventional biological removal (e.g. ibuprofen, 17β-estradiol and in some cases estrone and 17α-ethinylestradiol), whereas many others are moderately (e.g. sulfamethoxazole and metoprolol) or poorly (carbamazepine and venlafaxine) removed (Collado et al., 2014; Siegrist et al., 2005; Ternes, 1998). However, greater efficiencies have generally been demonstrated in CAS systems applying nitrification (McAdam et al., 2010; Ting and Praveena, 2017).

Indeed, many studies have demonstrated higher removals of PhACs in nitrifying activated sludge (NAS) systems (Fernandez-Fontaina et al., 2012; Tran et al., 2009; Yi and Harper, 2007). This enhanced biodegradation seems to be related to the activity of AOB which could co-metabolize these compounds using one of its key enzymes, AMO (Forrez et al., 2011). For example, Fernandez-Fontaina et al. (2014) reported that although sulfamethoxazole was slowly biodegradable (kbiol<1 L/gSS d) at μg/L concentration with autotrophic nitrifying biomass, its removal was enhanced at higher specific nitrification rates, this behavior being in accordance with the co-metabolic hypothesis and the fact that biotransformation of some compounds can rely on specific microbial populations.

As regards to EDCs, they are commonly removed by their direct use as electron donors by heterotrophs or via co-metabolism by AMO (Hamid and Eskicioglu, 2012). In a point of fact, the capability of pure AOB cultures, enriched AOB cultures and NAS systems has been suggested and a positive correlation was demonstrated between the activity of AOB and EDCs' degradation (De Gusseme et al., 2009; Forrez et al., 2008; Ren et al., 2007; Shi et al., 2004; Vader et al., 2000). Moreover, higher removals were reported under higher nitrification rates (Dytczak et al., 2008; Yi and Harper, 2007). On the other hand, an increasing number of studies have been acknowledging and reinforcing the importance of the heterotrophic community. Some authors demonstrated either the role of heterotrophic bacteria only (Bagnall et al., 2012) or the joint effect of both heterotrophs and AOBs that

could cooperatively enhance the elimination of EDCs (Khunjar et al., 2011; Ren et al., 2007).

In the case of AOB, this enhanced performance has been linked to the wide substrate range of AMO that has been found capable of oxidizing a large variety of pollutants simultaneously to the oxidation of NH_4^+, potentially being able to play a key role on several micropollutants' biodegradation (Tran et al., 2013). The contribution of AMO has normally been studied through the use of the inhibitor allylthiourea (ATU) that depletes the copper ions from its active site (Bédard and Knowles, 1989). Although there is a lack of understanding on how ATU interacts with other members of complex microbial communities (Men et al., 2017), it has widely been used in many studies to investigate the link between micropollutants biotransformation and nitrification (Fernandez-Fontaina et al., 2016; Ren et al., 2007; Roh et al., 2009; Sathyamoorthy et al., 2013; Tran et al., 2009).

At the same time, even though the potential of AOB has been widely studied, the potential of AOB in combination with anaerobic ammonium oxidizing (anammox) bacteria for the biodegradation of PhACs and EDCs has only been investigated in a limited number of studies and up-to-date results are somewhat contradictory. Removals comparable to those achieved in conventional BNR processes were obtained in two mainstream combined N/A systems for a group of micropollutants (including sulfamethoxazole, metoprolol, venlafaxine and carbamazepine) (Laureni et al., 2016), whereas very high removals (> 80%) were reported in one stage sidestream combined N/A process for estrone, estradiol, ethinylestradiol, naproxen, ibuprofen, bisphenol A and celestolide (Alvarino et al., 2015). These inconclusive results suggest the possible effect of different configurations (one or two stage reactors), operational conditions (optimal conditions for AOB and anammox in two stage reactors or moderate conditions in one stage reactor) and type of substrate (high or low strength NH_4^+ for sidestream or mainstream operation respectively), that could lead to diverse microbial dynamics and efficiencies.

From the aforementioned it is understood that the fate of PhACs and EDCs as well as the extent of their removal in both conventional and novel BNR processes are at the epicenter of current research, but up-to-date results on the efficiency of different technologies remain unclear. The expected stringent discharge limits in terms of emerging micropollutants' removal urge for optimization of the current methods and future developments must consider an integrated design approach to improve the overall WWTPs' efficiency. Thus, the

necessity for more in depth exploration in order to evaluate the contribution of the autotrophic and heterotrophic bacteria present in different engineered treatment systems towards the elimination of PhACs and EDCs, is being highlighted.

1.4 Compounds selected for the present thesis: properties, presence and removal

From the commonly used and studied pharmaceutical compounds that are present in the discharged effluents, some may pose a medium-high (acute) risk to aquatic life, whereas others have a low environmental risk but are discharged at high daily mass loads which could also contribute to negative effects on aquatic organisms in the long term due to chronic and mixture toxicities (Verlicchi et al., 2012). According to that, different PhACs and EDCs were here selected, based on their presence, persistence (linked to their low biodegradability) and environmental risk.

In the case of PhACs, the antibiotic sulfamethoxazole (SFX), the anti-inflammatory ibuprofen (IBU), the beta-blocker metoprolol (MTP), the anticonvulsant/antiepileptic carbamazepine (CBZ) and the antidepressant venlafaxine (VEN) were selected. SFX and IBU have been classified among those with the highest environmental risk, whereas CBZ and MTP pose lower risk but CBZ has the second higher average daily mass load (Verlicchi et al., 2012). Moreover, these compounds represent different therapeutical classes and they have different removal efficiencies: easily (IBU), moderate (SFX, MTP) and poorly (CBZ, VEN) biodegradable compounds (Collado et al., 2014, 2013, 2012; Rubirola et al., 2014).

As regards to EDCs, three natural estrogens namely estrone (E1), 17β-estradiol (E2) and estriol (E3), together with the synthetic contraceptive pill hormone 17α-ethinylestradiol (EE2) and the plasticizer bisphenol A (BPA) were selected. These compounds are reported as substances with high endocrine disrupting potency and have been linked to a wide variety of endocrine dysfunctions (De Gusseme et al., 2009; Diamanti-Kandarakis et al., 2009; Racz and Goel, 2010; Shi et al., 2004). Indeed, a framework evaluating the environmental risk of compounds originated from hospital and urban effluents stated that EE2 and E2 pose a high risk (risk quotient ≥ 1), whereas E1 and E3 have a risk in the medium-high region (risk quotient ≥ 0.1) (Al Aukidy et al., 2014). Additionally, different biodegradabilities have been reported, with E2 being the most biodegradable and EE2 along with BPA the least biodegradable compounds.

To be remarked that some of the chosen compounds (E1, E2 and EE2) are included in the watch list of Decision 2018/840/EU. Moreover, MTP, CBZ and VEN were proposed among the 12 indicator substances in order to monitor the removal efficiency of the Swiss WWTPs (http://www.esamur.com/public/file/McArdellSpainNov2016finalcopy.pdf).

The different properties of the studied compounds (i.e. volatility, absorbability-adsorbability and biodegradability), represented by different constants and coefficients (i.e. Henry's law constant-kH, solid-water partitioning coefficient-Kd and biodegradation rate constant-kbiol, respectively) as explained in Section 1.1.6, are presented in Table 1.1. Moreover, the molecular structures of the selected compounds are provided in SM (Tables S1 and S2).

Table 1.1: Properties of the studied PhACs and EDCs

Studied compounds	kH ($atm \cdot m^3$ /mole)	Log Kd	kbiol (L / gSS day)
IBU	7.83×10^{-8} [a]	0.9 [e] , 2.10 [f]	9 - 35 [l] , 1.33 - 35 [e]
SFX	7.84×10^{-11} [a]	0.3 [e] , 1.04 [f]	< 0.1 [l] , 2.1 - 2.6 [e]
MTP	1.26×10^{-7} [a]	1.2 [g]	0.35 - 0.40 [e]
CBZ	1.02×10^{-9} [a]	0.1 [e], 1.6 [i] , 1.[15 f]	< 0.01 [l] , < 0.005 - ≤ 0.1 [e]
VEN	2.04×10^{-11} [b]	1.9 [i]	–
E1	3.80×10^{-10} [c]	2.44 - 2.72 [j], 2.2 - 2.8 [d], 2.4 – 2.9 [e] , 2.45 [f]	200 - 300 [l] , 10 - 430 [e]
E2	3.64×10^{-11} [c]	2.45 - 2.83 [j], 2.41 - 2.84 [d] , 2.4 – 2.8 [e] , 2.56 [f]	300 - 800 [l] , 175 - 950 [e]
E3	1.33×10^{-12} -3.80×10^{-10} [c, d]	2.67 [f]	–
EE2	7.94×10^{-12} [c]	2.65 - 2.86 [j], 2.0 - 2.84 [d] , 2.5 – 2.8 [e] , 2.46 [f]	7 - 9 [l] , 0.4 - 20 [e]
BPA	1.26×10^{-7} [a]	4.37 [k]	0.5 - 73.7 [h]

[a] values predicted by the United States Environmental Protection Agency (EPA) (comptox.epa.gov/dashboard); [b] Rúa-Gómez and Püttmann (2012); [c] Silva et al. (2012); [d] Racz and Goel (2010); [e] Verlicchi et al. (2012); [f] Martín et al. (2012); [g] Götmar et al. (2000); [h] Clara et al. (2005); [i] Lajeunesse et al. (2013); [j] Hamid and Eskicioglu (2012); [k] Toro-Vélez et al. (2017); [l] Suárez et al. (2008)

Moreover typical ranges of concentrations (µg/L) in the influent and effluent of WWTPs, total removal (%), as well as the PNEC values and the potential risks that the selected compounds could pose to the environment are presented in Table 1.2.

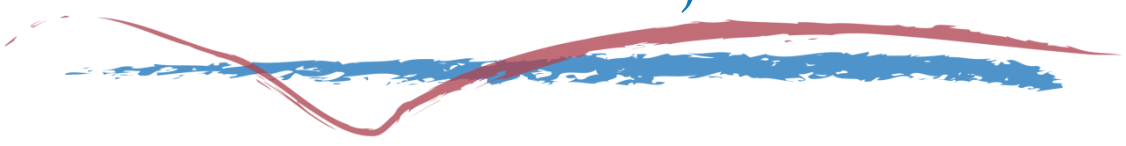
Table 1.2: Occurrence and fate of the selected PhACs and EDCs in WWTPs, PNEC values and risk quotients

Studied compounds	Influent (μg/L)	Effluent (μg/L)	Removal (%)	PNEC (μg/L)	Risk Quotient
IBU	0.0005 - 603 [a, b]	n.d.* - 55 [a]	72 - 100 [a, e, g]	1.65 [h, i] , 5 [a]	≈ 2 [h] , ≈3 [b]
SFX	< 0.003 - 10 [a, b]	< 0.003 - 1.15 [a]	4 - 88.9 [a, e, g]	0.027 [h] , 20 [a] , 0.15 [i]	10 [h, b]
MTP	0.002 - 1.52 [a, b]	0.003 - 0.25 [a]	3 - 56.4 [a, e, g]	8 [h]	0.04 [h] , ≈0.01 [b]
CBZ	0.0025 - 21.5 [a, b]	< 0.005 - 4.60 [a]	0 - 62.3 [a, e]	13.8 [h, i] , 25 [a]	0.08 [h] , ≈ 0.02 [b]
VEN	0.029 - 0.579 [c, d]	0.021 - 0.376 [c, d]	≈ 25 - ≈ 50 [f, d, e, g]	0.0057 [e]	–
E1	0.002 - 0.67 [a, b]	< 0.001 - 0.08 [a]	74.8 - 90.6 [a, e]	0.006 [b] , 0.018 [a] , 0.10 [i]	≈ 8 [b]
E2	0.002 - 3 [a, b]	< 0.001 - 0.007 [a]	90 - 100 [a, e]	0.002 [b], 0.01 [i]	≈ 4 [b]
E3	0.05 - 0.80 [a, b]	n.d. [a]	99 - 100 [a, e]	0.06 [b] , 0.149 [a], 1.52 [i]	≈ 1.5 [b]
EE2	0.001 - 0.04 [a, b]	< 0.001 - 0.002 [a]	43.8 - 100 [a, e]	0.0001 [b] , 0.00002 [a], 0.03 [i]	30 [b]
BPA	< 0.013 - 2.14 [a]	< 0.03 - 1.10 [a]	62.5 - 99.6 [a, e]	0.175 [e]	–

[a] Luo et al. (2014); [b] Al Aukidy et al. (2014); [c] Gros et al. (2012); [d] Baker and Kasprzyk-Hordern (2013); [e] Margot et al. (2015); [f] Rúa-Gómez and Püttmann (2012); [g] Collado et al. (2014); [h] Verlicchi et al. (2012); [i] Martín et al. (2012); n.d.: compound was not detected.

Risk Quotients (RQ) in the secondary effluent (average concentration/PNEC): 0.01 - <0.1 (low risk), ≥0.1 - <1 (medium risk), ≥1 (high risk).

Objectives



The present thesis sought to identify the potential of both conventional and novel BNR processes towards the simultaneous removal of N species, PhACs and EDCs, whereas the formation of main TPs was also evaluated. The followed experimental approach aimed to determine the contribution of the autotrophic and heterotrophic bacteria present in different lab, pilot and full-scale treatment systems performing: nitrification, partial nitrification (i.e. nitritation), combined partial nitritation-anammox and aerobic oxidation of COD.

A comparative assessment was carried out to determine which microbial groups are mainly involved in the elimination of the studied compounds. A range of clear intermediate objectives was set and distributed among the three chapters of which this thesis is constituted to unravel the removal capabilities of:

Enriched AOB biomass in the degradation of SFX

- Unravel the contribution of AOB on SFX degradation under increasing nitrification rates and in the presence of the inhibitor ATU. Establish the optimum conditions for higher efficiency.
- Determine the long-term biodegradation capacity of the system under a longer contact time and investigate possible acclimation factors and changes of the nitrification performance.
- Study the formation of SFX TPs and their further possible degradation by the enriched AOB culture.

Combined N/A biomass in the elimination of IBU, SFX, MTP, CBZ and VEN

- Unravel the contribution of the main autotrophic microbial groups (i.e. AOB and ANX) thriving in such a system in the presence of different substrates and/or the inhibitor ATU.
- Clarify the contribution of the heterotrophic fraction present in the sludge under optimal operating conditions.
- Study the formation and fate of some of the main TPs of the studied compounds.

Enriched nitrifying, AOB and conventional (mainly heterotrophic) biomass in the elimination of E1, E2, E3, EE2 and BPA

- Unravel the effect of increasing nitrification rates on EDCs elimination in the enriched NAS and AOB biomass.
- Determine the contribution of the heterotrophic bacteria and the small autotrophic fraction present in CAS in the presence of different combination of substrates and/or the inhibitor ATU.
- Study the correlation between E2 and E1.

THESIS OUTLINE:

Chapter I

Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products

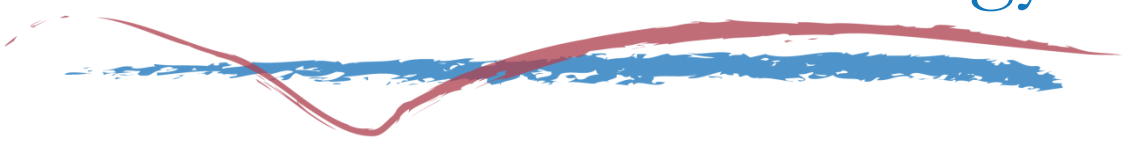
Chapter II

Unraveling the potential of a combined nitritation-anammox biomass towards the biodegradation of pharmaceutically active compounds

Chapter III

Comparative assessment of endocrine disrupting compounds removal in heterotrophic and enriched nitrifying biomass

Methodology



3.1 Description of lab-scale systems

Two fully automated sequencing batch reactors (SBR) with an active volume of 8 L were used for biomass cultivation in order to carry out part of the lab-scale experiments detailed in this thesis (Chapter I and III). Both SBRs were inoculated with activated sludge from a domestic WWTP located in Girona (Spain), but operated differently in order to promote partial nitrification (SBR-PN) and full nitrification (SBR-FN). Each SBR consisted of a stirrer, a feed, decant and purge pump, an aeration unit and sensors for pH (electrode 5333, CRISON) and dissolved oxygen (DO) combined with temperature control (InPro 6050 series sensor, Mettler Toledo). The SBRs were connected to a programmable logical controller (PLC, Siemens) and a SCADA (Omron Supervisor) system which enabled parameterized and automated operation.

3.1.1 SBR for partial nitrification (SBR-PN)

In this case, the enrichment of AOB was promoted and nitritation was achieved. The bioreactor was operated in cycles of 6 h, consisting of feed-1 (1 min), aeration-1 (120 min), feed-2 (1 min), aeration-2 (120 min), sludge wastage (2 min), settling (103 min) and decanting (15 min). The SBR-PN was fed with high strength synthetic reject wastewater (wastewater that simulates the effluent of an anaerobic digester in terms of NH_4^+ and bicarbonate concentrations), with the concentration at the influent being 1 g NH_4^+-N/L. One L of synthetic reject wastewater was added in each feeding period, while during the decant phase 2 L of the clarified supernatant were withdrawn. The HRT of the system was 24 h, whilst the SRT varied between 40 and 80 days in order to maintain the mixed liquor volatile suspended solids (MLVSS) concentration at c. 1 g/L. The temperature was controlled at 30^{o}C using a water jacket to mimic the common temperature conditions of reactors treating reject wastewater. Finally, DO was controlled between 0.5 and 3.0 mg O_2/L whereas pH was adjusted between 6.7 and 7.3 by adding 1 M $NaHCO_3$ solution.

The synthetic wastewater composition was modified from Kuai and Verstraete (1998) : 5640 mg/L of NH_4HCO_3 (1 g NH_4^+-N/L), 88 mg/L of KH_2PO_4, 110 mg/L K_2HPO_4 and 2 mL of trace element stock solution. The trace element stock solution consisted of (mg/L): 1250 EDTA, 550 $ZnSO_4 \cdot 7H_2O$, 400 $CoCl_2 \cdot 6H_2O$, 1270 $MnCl_2 \cdot 4H_2O$, 400 $CuSO_4 \cdot 5H_2O$, 50 $Na_2Mo_4 \cdot 2H_2O$, 1370 $CaCl_2 \cdot 2H_2O$, 1250 $FeCl_3 \cdot 6H_2O$ and 44400 $MgSO_4 \cdot 7H_2O$.

3.1.2 SBR for full nitrification (SBR-FN)

In this case, the enrichment of both AOB and NOB was promoted with the aim of establishing the whole nitrification process as found in conventional nitrifying plants at full scale. This bioreactor was operated under two different cycles. During the start-up period, the cycle consisted of feed (3 min), aeration-1 (240 min), sludge wastage (2 min), settling (95 min) and decanting (20 min). The long oxic phase assured the adaptation of the biomass to the higher NH_4^+ load. Moreover, a SRT of 50 days was established. Once the biomass was acclimated and equilibrium between both populations of interest had been achieved with all the NH_4^+ being converted to NO_3^- without NO_2^- accumulation, the second cycle was introduced and consisted of feed (3 min), aeration-1 (40 min), settling (81 min) and decanting (20 min). This shorter cycle created ideal conditions for faster growth. In this case the reactor was operated without sludge wastage in order to avoid losses of the slow-growing nitrifying bacteria and to develop the MLVSS concentration at c. 1g/L. Consequently, the SRT depended only on the sporadic sludge loss with the effluent and on solids sampling. The SBR-FN was fed with low strength synthetic NH_4^+ wastewater, with the concentration at the influent being 50 mg NH_4^+-N/L. Three L of synthetic wastewater were added in each feeding period, while during the decant phase 3 L of the clarified supernatant were withdrawn. The HRT of the system during the start-up period was around 16 h, whereas during the second cycle and after a steady state had been reached the reactor was operated at 6.4 h HRT. The mixed liquor temperature was controlled at 25 ^oC using a water jacket. Finally, DO was controlled between 0.5 and 3.0 mg O_2/L whereas pH was adjusted at 7.0 ± 0.3 by adding 1 M $NaHCO_3$ solution.

The synthetic wastewater composition consisted of : 282.1 mg/L of NH_4HCO_3 (50 mg NH_4^+-N/L), 87.8 mg/L of KH_2PO_4, 110.0 mg/L K_2HPO_4 (Castro-Barros et al., 2016) and 2 mL of trace element stock solution as previously described.

The batch experiments detailed in Chapter I and III were executed after complete adaptation of the biomass of both reactors had been obtained and a stable partial and full nitrification had been established in SBR-PN and SBR-FN, respectively.

3.2 Description of pilot and full-scale systems

Pilot-scale combined partial nitritation-anammox SBR (SBR-N/A)

A 400 L pilot-scale SBR of both attached (carrier: FLUOPUR® synthetic porous fleece material, WABAG Water Technology Ltd., Switzerland; 40% fill ratio) and suspended biomass was operated treating digester supernatant to promote combined N/A. The SBR-N/A consisted of a stirrer, a feed pump, a decanter, and an aeration unit and the process was controlled automatically by a PLC equipped with online sensors. The cycle comprised a feeding phase, an aeration phase, a mixing phase, a sedimentation phase, and a discharge phase. The SBR-N/A was operated by the Swiss Federal Institute of Aquatic Science and Technology (Eawag) and more details are described elsewhere (Joss et al., 2011).

Conventional WWTP

The WWTP of Quart (Spain) handles approximately 3000 population equivalents with a design flow of 600 m^3/d, mainly domestic, wastewater. It is designed for nitrogen (70 mg/L), phosphorus (20 mg/L) and chemical oxygen demand (COD) removal (495 mg/L). This facility consists of a conventional primary and secondary biological treatment. Average removal for COD is > 90%. CAS sludge was used to perform some of the batch experiments detailed in Chapter III.

3.3 Description of batch reactors

Different reactors were used in order to carry out the experiments detailed in Chapters I, II and III that are described below.

3.3.1 Batch experiments with biomass from SBR-PN

The batch experiments detailed in Chapter I were performed using a 1 L lab-scale Applikon stirred tank reactor coupled with a proportional-integral-derivative (PID) controller. Enriched AOB biomass was withdrawn from the SBR-PN during the settling phase previously sparged with compressed air for 5 minutes in order to oxidize any remaining NH_4^+. The biomass was subsequently washed with a phosphate buffer solution (PBS) with the following composition: 2.58 g/L $Na_2HPO_4 \cdot 12H_2O$, 0.44 g/L $NaH_2PO_4 \cdot 2H_2O$ and 7.54 g/L NaCl, in order to ensure that NO_2^- was removed completely. The initial mass of MLVSS at the beginning of all batch tests was 0.5 g. Aerobic conditions (>2.5 mg O_2/L) were achieved

with a continuous air supply. pH was automatically controlled during the experiments at 7.8±0.07 (by adding 0.6 M HCl or 1 M $NaHCO_3$ solution) and temperature was maintained at 29.7±0.6 °C.

Additionally, long term experiments directly in the SBR-PN were also carried out. During 10 weeks, SFX was added in the influent medium of the reactor (at different concentrations) in order to unravel if a longer contact time and possible acclimation of the biomass would affect its elimination.

3.3.2 Batch experiments with biomass from SBR-N/A

The batch experiments detailed in Chapter II were performed at lab temperature (≈ 25^{o}C) using a 12 L reactor that was controlled by a programmable logical controller (PLC) equipped with online sensors for water level, NH_4^+, NO_3^-, volumetric airflow control to the aeration unit, DO, temperature, pH, and conductivity. Prior to each experiment, 10 L of biomass were withdrawn from the SBR-N/A reactor and were adequately prepared in order to meet the necessary initial conditions in terms of substrate concentrations and DO levels depending on the aim of each test. The mixed liquor suspended solids (MLSS) concentration ranged during the batch experiments from 2.5 to 3.7 g/L, whereas regarding the attached biomass the fill ratio in the reactor was maintained at 40%. Finally, pH was controlled at 7.58 ± 0.24 by adding 1 M Na_2CO_3 or 0.6 M HCL.

3.3.3 Batch experiments with biomass from SBR-PN, SBR-FN and CAS

The batch experiments detailed in Chapter III were performed using Schott bottles that were placed on a multi-position magnetic stirrer at 200 rpm and at lab temperature (≈ 20^{o}C). Prior to each experiment, enriched NAS and AOB biomass was withdrawn from the SBR-FN and SBR-PN, respectively, during the aerobic phase. The biomass was subsequently washed with PBS as explained in 3.3.1, in order to ensure that NO_3^-, NO_2^- and any remaining NH_4^+ were removed completely. In the case of CAS, fresh biomass was taken the same day of the experiments from the aerobic basin of the WWTP of Girona. The CAS biomass was continuously sparged with air over a period of 2 h in order to assure complete oxidation of the organic matter present. Washed biomass was then used to seed each Schott bottle batch. The amount of biomass was adjusted in order to achieve an initial MLVSS concentration of approximately 0.5 g/L at a final volume of 200 mL. Aerobic conditions (>2.5 mg O_2/L) were

achieved with continuous air supply. pH was checked at regular time intervals and controlled between 7.3 and 7.6 by supplying 0.6 M HCL or 1 M $NaHCO_3$ solution.

3.4 Chemical characterization

3.4.1 Nutrients & Solids

ICRA laboratories

Mixed liquor samples were taken using a syringe and were filtered prior to analysis with 0.22 μm Millipore filter units for NH_4^+, NO_2^- and NO_3^-, and with 0.45 μm for acetate. NH_4^+, NO_2^- and NO_3^- were analyzed via ion chromatography (ICS5000, DIONEX.) and acetate via gas chromatography (Trace GC Ultra ThermoFisher Scientific). MLSS and MLVSS were analyzed according to the standard methods (APHA, 1998).

EAWAG laboratories

Mixed liquor samples were taken using a syringe and were filtered with 0.45 μm Macherey-Nagel filter units for NH_4^+, NO_2^- and NO_3^- and COD, prior to analysis. Since the reactor was of both attached and suspended growth, one part of the biomass was measured as suspended solids whereas another was measured as biofilm solids (carriers). The MLSS concentration was determined according to the standard methods (APHA, 1998) by the difference in weight of a dried filter (Macherey-Nagel Filter Paper Circles MN 640 90mm) without biomass and after heating at 105 ^oC for ≥ 24h and of the filter with biomass and after heating at 105 ^oC for ≥ 24h. Biofilm solids were determined by the difference in weight of dried carriers (105 ^oC for ≥ 24h) without biomass (unused ones) and with biomass (collected from the reactor). The concentration of NH_4^+ was analyzed using a flow injection analyzer (Foss FIA star 5000, Rellingen, Germany). The concentrations of NO_2^- and NO_3^- were analyzed by ion chromatography (Compact IC 761, Metrohm, Herisau, Switzerland). The concentration of COD was measured photometrically with test kits (Hach Lange, Düsseldorf, Germany).

3.4.2 Micropollutants

3.4.2.1 Pharmaceutically active compounds

Purchase

IBU, SFX, VEN and CBZ were purchased from Sigma-Aldrich. MTP, as MTP tartrate, was purchased from LGC Standards S.L.V. Regarding the TPs, 1-hydroxyl-IBU, 2-hydroxyl-IBU, Carboxyl-IBU, 4-NO_2-SFX, N^4-acetyl-SFX, o-desmethyl-MTP, α-hydroxyl-MTP, MTP acid, o-desmethyl-VEN, n-desmethyl-VEN, 2-hydroxyl-CBZ and 10,11-epoxy-CBZ were purchased from Toronto Research Chemicals. N-(5-Methyl-3-isoxazolyl) benzenesulfonamide (Desamino Sulfamethoxazole) was provided by Dr. Tobias Licha, from the Geoscience Centre of the University of Göttingen. Regarding the isotopically labeled compounds that were used as internal standards, ibuprofen-d3 was purchased from Sigma-Aldrich, carbamazepine-d10 and atenolol-d7 from CDN isotopes (Quebec, Canada), and finally sulfamethoxazole-d4 and venlafaxine-d6 from Toronto Research Chemicals (Ontario, Canada). HPLC-grade solvents water, methanol, acetonitrile (LiChrosolv) and 98% pure formic acid (HCOOH) were supplied by Merck (Darmstadt, Germany).

Sample preparation

Individual stock solutions were prepared on a weight basis, in methanol at 1 mg mL^{-1} and kept at -20 ^{o}C. Reference standard solutions as well as the calibration curve were prepared by appropriate dilution in methanol-water (10:90, v/v) of the stock solution of target compounds. Additionally, the samples collected over time were filtered through 0.45 μm Polyvinylidene fluoride membrane filters for PhACs (PVDF, Millipore).

- Batch experiments with biomass from SBR-PN

In the first study of this thesis (Chapter I) short term (6 h) as well as long term experiments (10 weeks) were conducted, and a different sample preparation was followed. During the short term experiments, the samples collected at time 0 and 30 minutes were diluted in methanol-water (10:90, v/v) to an appropriate concentration (the samples were diluted ten times in order to reassure that the results would be in the range of the calibration curve from 0.1 to 100 μg/L), while the others (time 3 and 6 hours) were not diluted. 10 μL of sulfamethoxazole-d4 (internal standard) at 1 mg/L was added to 1mL of each sample before injection in the Ultra Performance Liquid Chromatography tandem Mass Spectrometry

(UPLC-MS/MS) system. During the long term experiments, influent samples were diluted in methanol-water (10:90, v/v) to an appropriate concentration and analyzed by UPLC-MS/MS. Effluent samples were preconcentrated using solid phase extraction (SPE) in order to achieve proper sensitivity for the detection of potential TPs and metabolites of sulfamethoxazole. Oasis HLB cartridges (60 mg, 3 mL) were conditioned with 5 mL of methanol followed by 5 mL of HPLC grade water. 1.5 mL of a Na_2EDTA solution, having a concentration of 0.1M, was added to 25mL of effluent sample to achieve a final concentration of 0.1% (g solute/g solution) and loaded onto the cartridge. After sample pre-concentration, cartridges were rinsed with 6 mL of HPLC grade water and were dried with air for 5 min till total water removal. Analytes were eluted with 10 mL of pure methanol. Extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted with 1 mL of methanol/water (10:90, v/v). Finally, 10 μL of a 1 ng/μL of sulfamethoxazole-d4 at 1mg/L was added to 1mL of extract as internal standard and further analyzed by UPLC-MS/MS.

- Batch experiments with biomass from SBR-N/A

In the second study of this thesis (Chapter II) influent and effluent samples were preconcentrated using solid phase extraction (SPE) in order to achieve proper sensitivity for the detection of potential TPs of IBU, SFX, MTP, CBZ and VEN. The same protocol was followed as in the case of the long term experiments (described above), with the difference being the final volume of the samples which was 50 mL instead of 25. In this case, 10 μL of an internal standard mix (at 1 mg/L) were added to 1 mL of extract that was further analyzed by Ultra Performance Liquid Chromatography tandem Mass Spectrometry (UPLC-MS/MS).

Sample analysis: Ultra Performance TM-ESI-(QqLIT) MS/MS

All the samples from the experiments detailed in Section 3.3.1 and 3.3.2 were analyzed by UPLC (Waters Corp. Milford, MA, USA) coupled to a quadrupole-linear hybrid ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA). Chromatographic separation was carried out using different columns (all supplied from Waters Corp. Milford, MA, USA) and different chromatographic conditions as described in Table 3.1. For quantitative purposes, two MRM transitions were monitored for each compound, the first one for quantification and the second one (not available for all) for confirmation of the compound (Table 3..1). The data were recorded by using scheduled MRM^{TM} algorithm in order to increase sensitivity and selectivity. Data were acquired and processed using Analyst 1.5.1 software.

Table 3.1: Mass parameters for LC-MS/MS analysis.

			Quantification		Confirmation	
Compounds	**Rt (min)**	**Precursor ion (m/z)**	**Q1**	**DP/CE/CXP***	**Q3**	**DP/CE/CXP**
CBZ[a]	3.19	237 [M+H]+	194	61/29/28	193	61/49/14
CBZ-d10 (IS)	3.16	247 [M+H]+	204	46/31/32	--	--
10,11-epoxy-CBZ	2.88	253 [M+H]+	180	66/43/28	236	66/17/8
2- hydroxyl -CBZ	2.88	253 [M+H]+	210	76/27/12	208	76/33/12
VEN[a]	2.75	278 [M+H]+	58	66/55/10	260	66/17/12
VEN-d6 (IS)	2.74	284 [M+H]+	64	96/61/10	--	--
o-desmethyl-VEN	2.08	264 [M+H]+	58	151/57/10	77	151/83/12
n-desmethyl-VEN	2.70	264 [M+H]+	246	16/19/40	215	16/33/24
Chromatographic column used: Acquity HSS T3 (50 mm × 2.1 mm i.d., 1.8 µm particle size) in positive electrospray ionization mode						
IBU[b]	3.42	205 [M-H]-	161	-25/-10/-15	--	--
IBU-d3 (IS)	3.45	208 [M-H]-	164	-55/-10/-7--	--	--
1-hydroxyl-IBU	2.44	221 [M-H]-	177	-55/-10/-9	159	-55/-10/-13
2-hydroxyl-IBU	2.12	221 [M-H]-	177	-75/-12/-11	--	--
Carboxyl-IBU	0.92	235 [M-H]-	191	-20/-12/-7	73	-20/-22/-13
Chromatographic column used: Acquity BEH C18 (50 mm×2.1 mm i.d., 1.7 µm particle size) in negative electrospray ionization mode						
MTP[c]	3.3	268 [M+H]+	133	71/35/20	121	71/33/18
Atenolol-d7 (IS)	0.83	274 [M+H]+	145	61/37/10	--	--
MTP acid	1.6	268 [M+H]+	145	91/35/18	165	91/31/26
o-desmethyl-MTP	1.5	254 [M+H]+	177	76/25/10	133	76/35/20
α-hydroxyl-MTP	1.4	284 [M+H]+	207	76/27/30	119	76/37/16
Chromatographic column used: Acquity HSS T3 (50 mm × 2.1 mm i.d., 1.8 µm particle size) in positive electrospray ionization mode						
SFX[d]	1.5	254 [M+H]+	156	81/23/12	92	81/37/12
SFX-d4 (IS)	1.48	258 [M+H]+	160	101/23/18	--	--
N^4-Acetyl-SFX	1.6	296 [M+H]+	134	96/35/18	65	96/59/10
Desamino-SFX	1.9	239 [M+H]+	77	121/49/12	131	121/23/10
4- NO_2-SFX	2.1	284 [M+H]+	92	111/77/12	189	111/37/8
Chromatographic column used: Acquity BEH C_{18} (50 mm × 2.1 mm i.d., 1.7 µm particle size) in positive electrospray ionization mode						

*CE: collision energy, DP: declustering potential, , CXP: collision cell exit potential

[a]Gros et al. (2012); [b]Ferrando-climent et al. (2012); [c]Rubirola et al. (2014); [d]Present thesis Chapter I)

The limits of detection (LOD) and quantification (LOQ) are presented in Table 3.2. In case of concentrations below LOQ or LOD, the LOQ or LOD values themselves were considered for

calculation purposes. The analytical error for the compounds, based on the performed recovery (average values between 108% and 123%), was between 4 and 5%.

Table 3.2: LOD and LOQ of the studied PhACs

Compound	LOD (µg/L)	LOQ (µg/L)
CBZ	0.02	0.08
10,11-epoxy-CBZ	0.01	0.05
2- hydroxyl-CBZ	0.02	0.04
IBU	0.08	0.27
1-hydroxyl-IBU	0.22	0.73
2-hydroxyl-IBU	0.37	1.24
Carboxyl-IBU	n.r.	n.r.
MTP	0.01	0.05
o-desmethyl-MTP	0.01	0.02
MTP acid	n.r.	n.r.
α-hydroxyl-MTP	0.02	0.06
SFX	0.01	0.05
4- NO_2-SFX	0.26	0.87
Desamino-SFX	0.02	0.07
N^4-Acetyl-SFX	0.02	0.07
VEN	0.01	0.02
o-desmethyl-VEN	0.03	0.09
n-desmethyl-VEN	0.11	0.37
n.r.: not reported		

3.4.2.2 Endocrine disrupting compounds

Purchase

E1, E2, E3, EE2 and BPA were purchased from Sigma-Aldrich. Regarding the isotopically labeled compounds that were used as internal standards, E1-2,4,16,16-D4 (E1-d4), E2-17β-2,4-D2 (E2-d2), EE2-17α-2,4,16,16-D4 (EE2-d4) and BPA-2,2',6,6'-d4 (BPA-d4) were obtained from CLUZEAU INFO LABO (C.I.L.).

Sample preparation

- Batch experiments with biomass from SBR-PN, SBR-FN and CAS

In the third study of this thesis (Chapter III), all the samples collected along the batch experiments were filtered through 0.45 μm PVDF filters and stored at 4^{o}C for 1 day prior to analysis. An internal standard containing BPA-d4, E1-d4, E2-d2, EE2-d4 at a concentration of 1 mg/L each, was prepared (E3 and E2 shared the same internal standard). 50 μL of the internal standard mix were added in 1 mL of each sample before injection in the Ultra Performance Liquid Chromatography tandem Mass Spectrometry (UPLC-MS/MS) system. The calibration curve was prepared by appropriate dilution of the stock solution in methanol: water (15:85, v/v).

Sample analysis: Ultra High Performance Liquid ChromatographyTandem Mass Spectometry

Samples were analyzed by an Ultra Performance Liquid Chromatography (Thermo Fisher Scientific) system coupled to a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific). Chromatographic separation was carried out using a LUNA OMEGA C_{18} 1.6 μm (100 × 2.1 mm) (Phenomenex) column. The separation conditions in the mobile phase were as follows: solvent (A) methanol, solvent (B) UHPLC water at a flow rate of 400 μL/min. The gradient elution was: initial, 20% of solvent A; 0-1.75 min, 20-50% of solvent A; 1.75-5.5 min, 50-100% of solvent B; 5.5-8.0 min, 100% B; 8.0-9.5, return to the initial conditions; 9.5-10.5 min, equilibration of the column. Based on the characteristics of the selected compounds the mass spectrometer ion spray source (ESI) was operated in negative mode. Optimal source parameters were: capillary temperature = 300^{o}C, spray voltage = 2500 V, vaporizer temperature = 350^{o}C, sheath gas pressure = 40 and auxiliary gas pressure = 20. From the different mass spectrometry acquisition methods, the Selected Reaction Monitoring (SRM) was used, in which an ion of a particular mass (precursor ion) is selected in the first spectrometer stage and a second ion, product of a fragmentation reaction of the precursor ion is selected in the second stage for detection. Two transitions were monitored for each compound, the first one for quantification and the second one for confirmation of the compound (Table 3.3). Data were acquired using Xcalibur 2.2. program and processed using TraceFinder 3.1 software.

Table 3.3: Optimized MS/MS parameters for target compounds

Compound	Precursor 1 (m/z)	SRM 1	CE* 1	SRM 2	CE* 2
BPA	227	212	21	133	27
E1	269	145	38	143	54
E2	271	183	42	145	43
E3	287	171	41	145	40
EE2	295	145	41	159	33
BPA-d4	231	216	19	–	–
E1-d4	273	145	40	–	–
E2-d2	273	147	43	–	–
EE2-d4	299	147	41	–	–

*CE: collision energy

The LOD and LOQ of the studied EDCs for each of the three types of biomass investigated are presented in Table 3.4. In case of concentrations below LOQ or LOD, the LOQ or LOD values themselves were considered for calculation purposes. In this case, no recovery was performed as a direct injection was applied.

Table 3.4: LOD and LOQ of the studied EDCs

	SBR-PN		SBR-FN		CAS	
	LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)
BPA	0.12	0.40	0.05	0.18	0.07	0.24
E1	0.02	0.07	0.00	0.02	0.01	0.03
E2	0.04	0.15	0.01	0.05	0.02	0.05
E3	0.02	0.06	0.03	0.09	0.01	0.05
EE2	0.05	0.15	0.02	0.07	0.06	0.21

3.5 Microbial characterization

Different analyses of bacterial populations were performed in this thesis. Fluorescence in situ hybridization (FISH) was carried out in the first study (Chapter I), in order to evaluate the AOB and NOB communities' enrichment. In the second study (Chapter II), the characterization of the bacterial communities present in the combined N/A pilot-scale reactor was assessed by amplicon-targeted sequencing of bacterial 16S rRNA gene using Illumina chemistry (Miseq, 2x250 paired-end).

3.5.1 Fluroscence in situ Hybridization

FISH analysis was conducted on the biomass from the SBR-PN at the time of the study to assess AOB abundance. FISH was performed as described in (Nielsen et al., 2009) with Cy5-labeled EUBMIX probes (for general bacteria) and Cy3-labeled AOBMIX probes (for AOBs, comprising equal amounts of probes Nso1225, NEU, NmV, Cluster6a192) and Cy3-labeled Nso190. FISH preparations were visualized with a Nikon CS1 confocal laser-scanning microscope (CLSM) using Plan-Apochromat 63_oil (NA1.4) objective. Thirty images were taken from each sample for quantification. The area containing Cy3-labeled specific probe (AOBMIX + Nso190 for AOB) cells was quantified proportionally to the area of Cy5-labeled bacteria probe (EUBMIX) within each image using the daime software package (Daims et al., 2006).

3.5.2 Gene sequencing with the Illumina MiSeq System

DNA extraction and high-throughput sequencing

Samples of both homogenized mixed liquid and carriers were collected from the 400 L pilot-scale reactor and immediately frozen at -30ºC until DNA extraction. As a first step, a specific protocol was followed to extract the biomass from the carriers and obtain a pellet, as in the case of the mixed liquor sample. Ten carriers were placed in a vial and covered with 3 mL of Sodium Phosphate Buffer and 122 µL of MT Buffer. The vial was placed in a desktop ultrasonic cleaning bath for 15 minutes (P-Selecta, BCN, Spain), and the biomass was recovered in the liquid phase. The sample was then centrifuged at 11.000 rpm for 15 minutes in an Eppendorf 5424 centrifuge (Eppendorf AG, Hamburg, Germany). DNA extraction for both types of samples (biomass-pellet and carrier-pellet) was then performed using FastDNA® Spin kit for Soil (MP Biomedicals, Santa Ana, California, USA) according to manufacturer instructions. DNA extracts were quantified using a Qubit® 2.0 Fluorometer (Invitrogen Molecular probes Inc., Oslo, Norway).

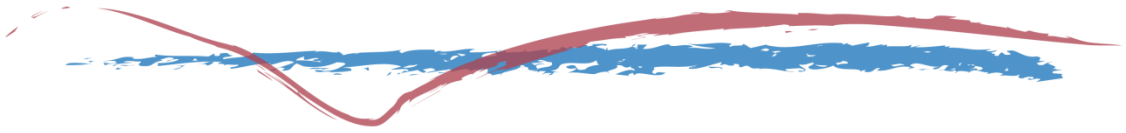
High-throughput multiplexed 16S rRNA gene sequencing with the Illumina MiSeq System (2×250 PE) was carried out using primer pair 515f/806r (Caporaso et al., 2011) targeting the V4 region of the 16S rRNA gene complemented with Illumina adapters and sample-specific barcodes at the genomics core facilities and methods of the Research Technology Support Facility Michigan State University, USA (Kozich et al., 2013). Raw forward and reverse paired sequences were merged and quality filtered using the UPARSE pipeline implemented

in USEARCH (Edgar, 2013). De-novo and reference-based chimera checking, Operational Taxonomic Unit (OTU) clustering (97% cutoff) removing singletons, identification of representative OTU sequences and construction of OTU table were also carried out in USEARCH using the UPARSE-OTU algorithm. The resulting OTU table was converted to Biological Observation Matrix (BIOM) format (McDonald et al., 2012) and then imported and analyzed into QIIME (Caporaso et al., 2010a). Sequencing depth ranged between 29,792 and 41,153 sequences per sample after removing non-bacterial, unspecific reads (i.e. archaeal and unclassified sequences that constituted 0.9% and 0.6% of total reads, respectively). In QIIME, representative sequences from each OTU were aligned to the Greengenes imputed core reference alignment (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010b). Taxonomical assignments for each OTU were done using the BLAST method and the QIIME-formatted version of the SILVA 119 reference database (Quast et al., 2013). For community analysis, the number of sequences in each sample was normalized by randomly selecting a subset of 29,000 sequences from each sample to standardize sequencing effort across samples and to minimize bias due to different number of total sequences. QIIME was also used to calculate the Shannon index of diversity (alfa diversity) and to compare microbial communities in the different samples (beta diversity) according to the weighted UniFrac distance (Lozupone and Knight, 2005). In the latter case, differences were assessed for statistical significance using the PERMANOVA test implemented in QIIME. The sequence data set was deposited in the NCBI Sequence Read Archive (SRA) database under accession nº: SRP144938.

Block II

Results

Chapter I



"Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products"

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4.1 Preliminary remarks

The aim of the present study was to explore the biodegradation capacity of an enriched AOB culture (> 80% of the microbial population belonging to the AOB group) towards SFX. The biomass was cultivated in the SBR-PN (described in Section 3.1.1) treating high strength synthetic reject wastewater (1 g NH_4^+-N/L). Short term and long term experiments were carried out, with the aim of demonstrating if AOB were able to degrade SFX (initial design concentration of 100 μg/L) and if so, under which conditions. On one hand, several short term (batch) experiments were performed under different conditions in order to evaluate the effect of the nitrification rate measured as specific NH_4^+ oxidation rate (SAOR), the role of AMO enzyme and the effect of adding a carbon source in the nitrifying culture, on SFX degradation. Additionally, on the other hand, long term experiments (10 weeks) were carried out directly in the SBR-PN, so as to investigate the effect of a longer contact time and of possible acclimation factors in the removal of SFX (at 10 and 100 μg/L design influent concentrations). Finally, the formation of two TPs: 4-NO_2-SFX and Desamino-SFX as well as of a human metabolite: N^4-Acetyl-SFX, and their correlation with the parent compound were also investigated.

4.2 Materials and methods

4.2.1 Bioreactor set-up and operation

An 8L SBR was inoculated with activated sludge from a domestic WWTP located in Girona (Spain). The reactor was operated in order to promote partial nitrification and to develop an enriched AOB culture. Cycle studies were performed on a weekly basis to monitor the nitrification activity in the SBR-PN, and samples for the analyses of NH_4^+, NO_2^- and NO_3^- were taken along the cycle. At the end of the aerobic phase MLSS and MLVSS solids were also analyzed. More details on the set-up and operation of the reactor as well as on the chemical characterization are provided in Section 3.1.1 and 3.4.

The experiments detailed in the present Chapter were conducted after more than 1 year of reactor operation, with a stable AOB population (more than 80% of the total microbial community belonging to the AOB group as demonstrated in the FISH micrographs, Figure S1

in supplementary material-SM) and with stable nitritation performance (95% of NH_4^+ converted to NO_2^- and no NO_3^- detected in the effluent).

4.2.2 Batch experiments

Different sets of tests were performed (Table 4.1) with a duration of 6 hours. During all tests NH_4^+ was continuously dosed for the first 4 hours at a loading rate of 0.002 L/min to ensure a constant ammonia oxidizing rate. The last 2 hours were monitored to investigate if and how the lack of NH_4^+ would affect the removal of SFX.

The fed NH_4^+ in T1-T19 (Table 4.1, first category) was in the range 69.5 - 481.3 mg NH_4^+-N/L, leading to NH_4^+ loading rates from 0.12 to 0.96 mg/min and to SAORs (that represent the amount of ammonia oxidized during the first 4 hours of the experiment per sludge mass and time) from 0.25 to 2.1 mg NH_4^+-N/(g MLVSS min). T20 and T21 were executed in the absence of NH_4^+ and therefore with SAORs of 0 mg NH_4^+-N/(g MLVSS min). In T22-T25 (Table 4.1, second category) ATU was spiked (25 mg) at the beginning of the test to suppress the degradation of SFX by NH_4^+ oxidation. Likewise, in T24-T29 100 mg of acetate was spiked to investigate the contribution of the heterotrophic fraction of the biomass on SFX degradation. All T1-T29 tests had an initial SFX mass of 100 μg. Finally, T30-T35 experiments (Table 4.1, third category) were conducted adding 4- NO_2-SFX, Desamino-SFX and N^4-acetyl-SFX separately (initial spiked TP mass of 100 μg), without any addition of SFX.

Samples were taken at the beginning of the test, after half an hour, after three hours and at the end of the test (6 h) for chemical analyses of nutrients, SFX and SFX TPs. MLSS and MLVSS were measured at the beginning and at the end of each test. The working volume of the batch reactor increased from 0.5L at the beginning up to 1L at the end of the tests due to the continuous and constant NH_4^+ supply (when applicable); therefore the results presented refer to masses.

Additionally, a sorption control experiment was performed with SFX and autoclaved biomass to assess possible losses (Figure S2 in SM). Moreover, preliminary abiotic experiments were executed with SFX at different concentrations of NO_2^-, aiming to investigate possible chemical transformations under different sample preservation conditions (at -20^{o}C and at 4^{o}C, overnight). It was confirmed that neither chemical transformation of SFX nor chemical

formation of 4- NO_2-SFX can occur in the presence of NO_2^-, in none of the cases (Figure S3 in SM). More details regarding the experimental conditions are provided in SM.

Table 4.1: List of conducted experiments with AOB and SFX

Category	Code	NH_4^+-N load[a]	SAOR	SFX[b]	ATU[b]	ACE[b]	4-NO_2[b]	N^4-Acetyl[b]	Desamino[b]
1) NH_4^++SFX	T1	0.96	2.02	100	--	--	--	--	--
	T2	0.93	2.10	100	--	--	--	--	--
	T3	0.90	1.66	100	--	--	--	--	--
	T4	0.87	1.72	100	--	--	--	--	--
	T5	0.78	1.86	100	--	--	--	--	--
	T6	0.60	1.18	100	--	--	--	--	--
	T7	0.60	1.04	100	--	--	--	--	--
	T8	0.56	1.08	100	--	--	--	--	--
	T9	0.39	0.77	100	--	--	--	--	--
	T10	0.37	0.70	100	--	--	--	--	--
	T11	0.36	0.70	100	--	--	--	--	--
	T12	0.29	0.59	100	--	--	--	--	--
	T13	0.28	0.56	100	--	--	--	--	--
	T14	0.26	0.61	100	--	--	--	--	--
	T15	0.26	0.52	100	--	--	--	--	--
	T16	0.19	0.43	100	--	--	--	--	--
	T17	0.18	0.39	100	--	--	--	--	--
	T18	0.14	0.28	100	--	--	--	--	--
	T19	0.12	0.25	100	--	--	--	--	--
	T20	0	0	100	--	--	--	--	--
	T21	0	0	100	--	--	--	--	--
2) NH_4^++SFX + ATU/ACE	T22	0.61	0.11	100	25	--	--	--	--
	T23	0.54	0.15	100	25	--	--	--	--
	T24	0.65	0.04	100	25	100	--	--	--
	T25	0.62	0.01	100	25	100	--	--	--
	T26	0.28	0.64	100	--	100	--	--	--
	T27	0.25	0.54	100	--	100	--	--	--
	T28	0	0	100	--	100	--	--	--
	T29	0	0	100	--	100	--	--	--
3) TP	T30	1.04	2.18	--	--	--	100	--	--
	T31	0.96	2.07	--	--	--	100	--	--
	T32	1.02	2.15	--	--	--	--	100	--
	T33	0.99	2.24	--	--	--	--	100	--
	T34	0.96	1.76	--	--	--	--	--	100
	T35	0.88	1.21	--	--	--	--	--	100

Units: NH_4^+-N load (mg/min); SAOR (mg NH_4^+-N/g MLVSS min); SFX and its TPs (4-NO_2-SFX, N^4-Acetyl-SFX and Desamino-SFX) (μg); ATU and ACE (mg)

[a] NH_4^+-N load equals to ammonia oxidation rate in the batch reactor since no accumulation of ammonia was observed.

[b] The values refer to the theoretical masses added in the system.

*In the tables and figures of the present thesis the acronym ACE stands for acetate.

4.2.3 Long term experiments

The removal capability of the enriched AOB population present in the nitritation SBR was investigated under a longer contact time of 24 h (since the HRT of the system was 1 day) and in long term conditions. The SBR-PN was operated for 10 weeks with the presence of SFX in its influent following the same cycle previously described in Section 3.1.1. The removal efficiency of the SBR-PN towards SFX was investigated, at constant MLVSS (2g/L), first at an influent concentration of 10 µg/L for five weeks (days 1-35), and then of 100 µg/L during the five following weeks (days 36-70).

The influent medium was sampled every day (i.e. exactly after the completion of 4 cycles) to ascertain that the concentration of SFX remained stable and to ensure that no abiotic SFX degradation was occurring (influent sample). The SBR-PN effluent was collected in a tank and a composite 24 hours sample was taken on a daily basis (effluent sample) and was correlated to the influent sample of the previous day for removal calculations. Both influent and effluent samples were collected at the same time for chemical analyses of nutrients, SFX and SFX TPs (4-NO_2-SFX, Desamino-SFX and N^4-acetyl-SFX). Long term SBR-PN experiments were executed after all batch experiments described in Section 4.2.2 were completed to avoid any inconsistency attributable to the potential biomass adaptation to SFX.

4.3 Outcomes

4.3.1 Batch experiments

4.3.1.1 SFX degradation and TPs formation at different SAORs

The degradation of SFX and the formation of 4-NO_2-SFX (4-NO_2-SFX), Desamino-SFX and N^4-Acetyl-SFX were evaluated in all the experiments. A time course of the amounts of SFX, its TPs and the obtained SAORs are shown for three representative tests (T4, T13 and T17) in Figure 4.1. Also, time course data for all the experiments carried out in this study (T1-T35) is provided in SM (Figures S4-S13). NH_4^+ was oxidized as soon as it entered the system and no accumulation was detected; NO_2^- was increasing throughout the tests as expected and NO_3^- was not detected in any of the experiments.

SFX experienced, at higher SAORs, a sharp decrease during the first half an hour, with a lower decrease rate in the rest of the experiment (Figure 4.1a and b). At a lower SAOR, SFX

removal was significantly affected and, after a first moderate decrease, no further removal was detected (Figure 4.1c). 4-NO_2-SFX was formed in all the tests but the ones with ATU (T22-T25), whereas the formation of Desamino-SFX and N^4-Acetyl-SFX was negligible in most of the cases. Moreover, a correlation was found between SFX degradation and 4-NO_2-SFX formation, as discussed later.

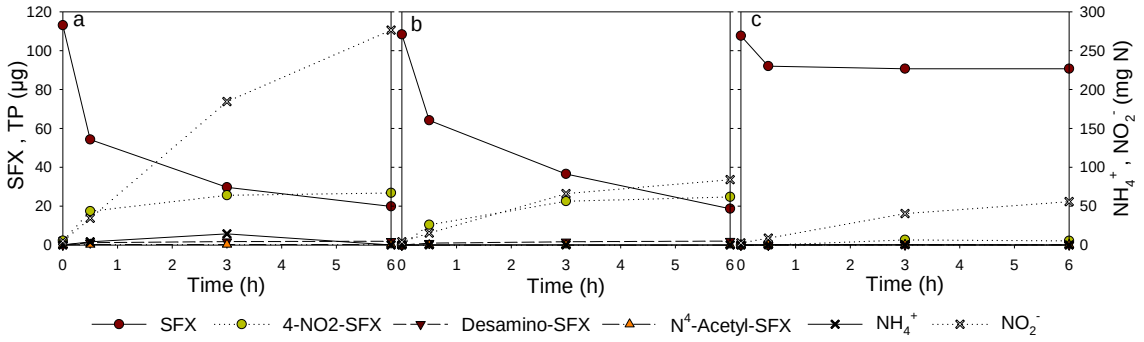


Figure 4.1: Removal of SFX (µg) and TPs formation under different SAOR rates: a) 1.72 mg NH_4^+-N/(g MLVSS min); b) 0.56 mg NH_4^+-N/(g MLVSS min); c) 0.39 mg NH_4^+-N/(g MLVSS min).

In the first category of tests (T1-T21) different NH_4^+ loading rates were applied in the system, thus different SAORs were obtained. SFX degradation was clearly related to the nitrification rate since higher removals were observed at higher specific NH_4^+ oxidation rates (Figure 4.2a). When nitrification did not occur due to the lack of ammonia, SFX degradation was still observed at around 25%. Based on the range of SAORs that are obtained, three different levels of removal (low, moderate or high) can be expected, as identified in the present study: i) $0 \leq$ SAOR ≤ 0.4 mg NH_4^+-N/(g MLVSS min) (T16-T21) corresponding to a range of limited removals of 16-33% (average 25±7%); ii) $0.5 \leq$ SAOR ≤ 1.2 mg NH_4^+-N/(g MLVSS min) (T6-T15) corresponding to a range of removals of 53-83% (average 67±12%); and iii) $1.6 \leq$ SAOR ≤ 2.1 mg NH_4^+-N/(g MLVSS min) (T1-T5) corresponding to a range of high removals of 79-91% (average 86±5%) (Figure 4.2b).

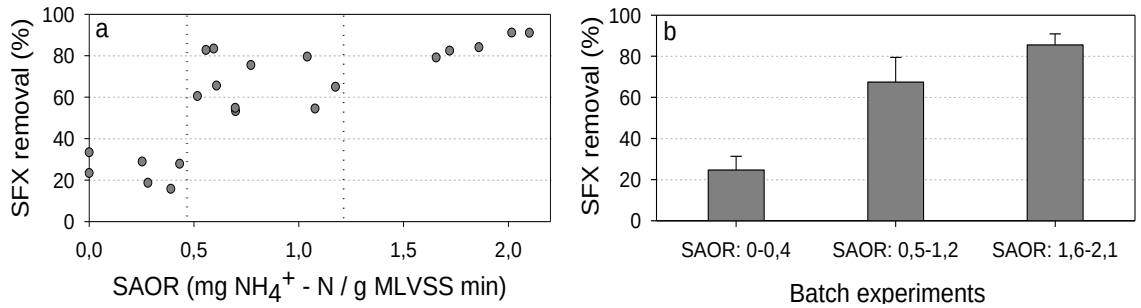


Figure 4.2: a) Removal of SFX (%) as a function of the investigated SAOR rates (T1-T21) (vertical dotted lines represent the 3 different degradation zones defined); b) Average removal of SFX (%) for the three identified zones. The error bars represent the standard deviation.

Different batch tests were executed adding ATU and acetate with the intention of clarifying the contribution to SFX degradation of the autotrophic and of the small heterotrophic fractions present in the sludge. The effect of both compounds was investigated independently as well as in combination (Figure 4.3).

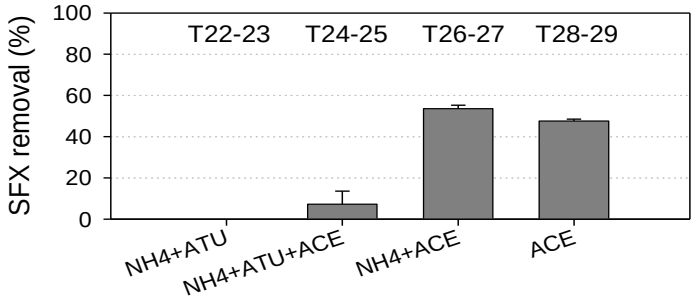


Figure 4.3: Effect of ATU and acetate on SFX degradation (average removal presented as percentage). The error bars represent the standard deviation.

In the presence of ATU (T22-T23), nitrification was completely suppressed and this resulted in a null removal of SFX, providing a first indication of the role of the AOBs in the total degradation. Interestingly, when acetate was spiked in the system, in the presence (T26-T27) and absence (T28-T29) of NH_4^+, similar SFX removals of 54% and 48% were observed respectively with an average acetate consumption of 17.8 ± 8.2%. However, in the tests that were executed in the presence of both ATU and acetate (T24-T25), the removal of SFX decreased to very low levels (7.3±6.3%), suggesting a connection between AMO and SFX degradation.

4.3.1.2 Formation of TPs

An efficient UPLC-QqLIT method for the determination of 4-NO_2 SFX, Desamino-SFX and N^4-Acetyl-SFX in batch samples without sample pre-concentration was optimized and validated with good quality parameters. To the extent of our knowledge this is the first study on AOB enriched biomass evaluating these three TPs (more details regarding the analytical technique are provided in SM.).

The average formations of 4-NO_2-SFX, Desamino-SFX and N^4-Acetyl-SFX were evaluated in parallel to SFX degradation in all the batch experiments. TPs formation represented up to an average of 30% of the initial SFX mass (Figure 4.4). 4-NO_2-SFX was detected in almost all tests. Moreover, it was the main detected TP since the difference between its formation and the total formation of all the TPs was significantly low. The results of the first category of experiments executed with NH_4^+ and SFX (T1-T21) demonstrated that nitrification rate not only affected SFX degradation, but also the formation of the TPs. Considering the three SAOR zones previously described, higher TP formation was quantified for higher SAORs. Moreover, higher TP formation was observed for higher SFX degradation, except in the case of the tests T28-T29, where an intermediate removal of SFX led to null formation of TPs. As a matter of fact, in the presence of acetate (T24-T25, T26-T27 and T28-T29), 4-NO_2-SFX was not formed to the same extent as compared to the rest of the experiments. During T24-25 and T28-29 no accumulation of this TP was observed despite the detected SFX removal, whereas a formation around 20% was observed in experiment T26-27. This could lead to the hypothesis that higher concentrations of NO_2^- (like in the case of experiments T26-T27 in contrast to T24-T25 and T28-T29) can lead to higher formation of 4-NO_2-SFX. However, on one hand, NO_2^- was measured in the bulk liquid in all of these experiments at an order of magnitude higher than SFX (minimum concentration around 5 mg/L), while on the other hand, chemical transformation of this compound was ruled out (as demonstrated in preliminary abiotic experiments). Results suggest the potential contribution of the heterotrophic bacteria. However, although these findings seem promising, more systematic studies are needed in order to corroborate the role of solely heterotrophs or both heterotrophs and nitrifiers in the elimination of 4-NO_2 and other SFX TPs. Finally, in the case that SFX was not degraded due to the addition ATU (T22-T23), no formation of TPs was detected.

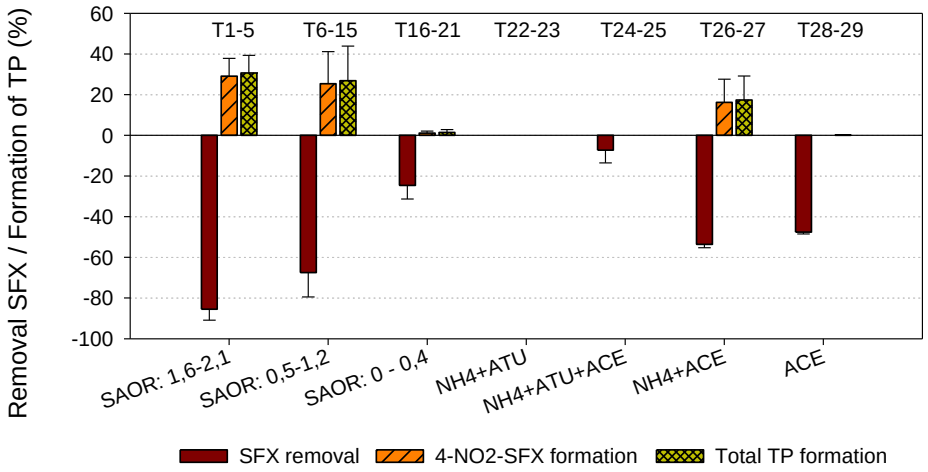


Figure 4.4: Removal of SFX and TPs production for all the conducted tests. The error bars represent the standard deviation.

The fate of SFX TPs was also explored by means of independent experiments for each of them, and results are shown for three representative tests (T31, T32 and T34) in Figure 4.5. The compounds showed a relatively stable behavior during the experiments with a limited increase during the first 30 minutes for 4-NO_2-SFX and Desamino-SFX (Figure 4.5a and b, respectively), possibly for an insufficient mixing at time zero, considering that no significant increase was observed from then onwards. N^4-Acetyl-SFX remained stable during all the experiment (Figure 4.5c).

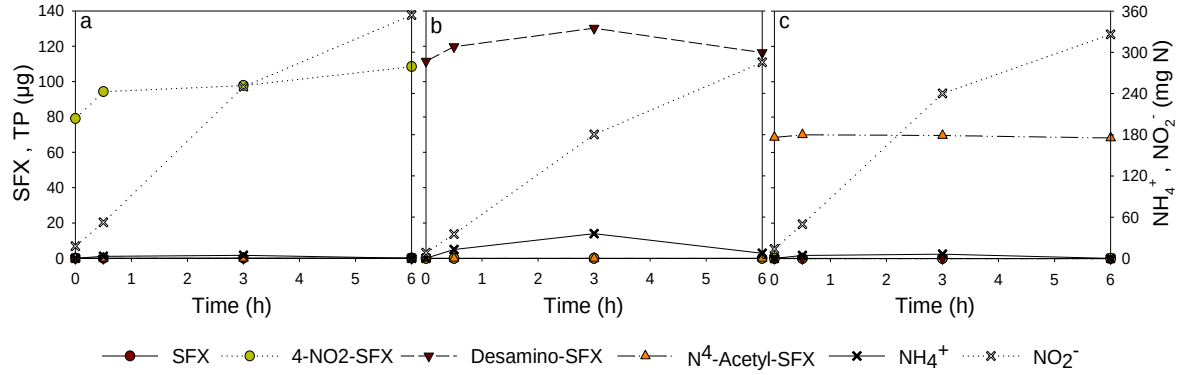


Figure 4.5: Fate of TPs during the independent experiments: a) 4-NO_2-SFX (T31); b) Desamino-SFX (T34); c) N^4-Acetyl-SFX (T32).

In all tests, the only detected TP was the one spiked. Moreover, it is important to stress that TPs were not cleaved back to SFX and that they could not be degraded during the batch tests.

4.3.2 Long term experiments

4.3.2.1 SFX degradation and TPs formation during the long term test in the SBR-PN

The performance of the SBR-PN was kept stable during the 10 weeks of the long term experiment. The addition of SFX in the influent of the reactor did not affect the nitritation performance (data not shown). In fact the reactor achieved more than 99% of ammonia removal. No NO_3^- was detected in the effluent.

During the first period (1-35 days), the concentration of SFX at the influent was 11.2 ± 0.8 µg/L whereas during the second period (36-70 days), the influent SFX concentration was 101.7 ± 12.2 µg/L. High SFX removals were observed at both influent concentrations with the SBR-PN operating at a SAOR of 0.5 mg NH_4^+-N/(g MLVSS min). SFX was rapidly degraded in the system, without a previous period of acclimation of the biomass, and from the first day a removal around 98% was obtained. During the first period, at the lowest influent concentration, SFX removal was 98.3 ± 3.2% (effluent concentration of 0.2 ± 0.3 µg/L, Figure 4.6a). During the second period, at the highest influent concentration, the removal of SFX was 97.6 ± 3.2% (effluent concentration of 2.5 ± 3.5 µg/L, Figure 4.6b).

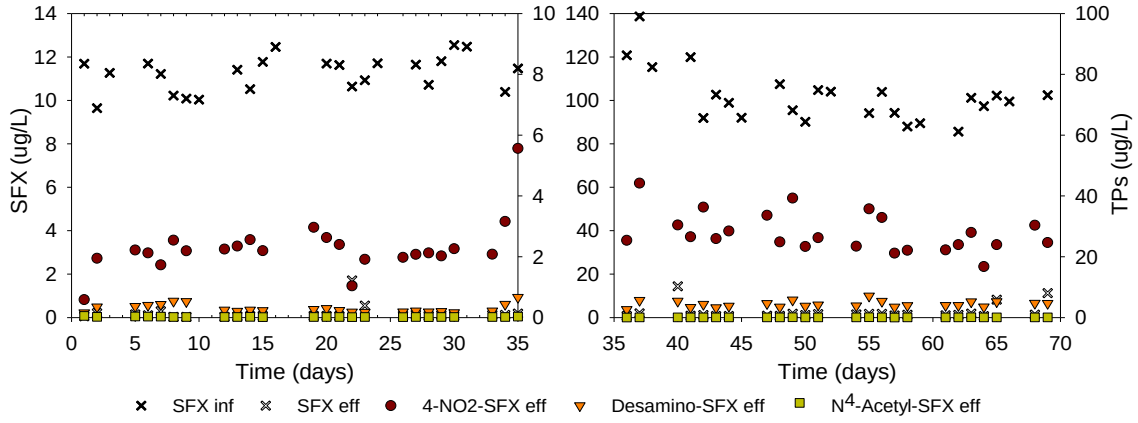


Figure 4.6: Removal of SFX and TPs formation during the long term experiment executed in the SBR-PN: a) day 1-35, influent SFX concentration of 11.2 ± 0.8 µg/L; b) day 36-70 influent SFX concentration of 101.7 ± 12.2 µg/L.

In terms of TPs, a similar behaviour was observed as in the case of the batch tests. 4-NO_2-SFX was detected in all tests and was the main TP. 4-NO_2-SFX was formed up to 20.5 ± 7.8% (effluent concentration of 2.3 ± 0.9 µg/L), and up to 27.6 ± 6.1% (effluent concentration of 27.9 ± 6.4 µg/L) during the first (1-35 days) and second (36-70 days) period respectively. The total TPs formation ranged from 23.4 ± 8.7% to 32 ± 7% during the first and the second

period respectively. Adversely, in this experiment Desamino-SFX and N^4-Acetyl-SFX formation remained at low levels compared to 4-NO_2-SFX, but both were detected in the system. Desamino-SFX formation ranged from 2.7 ± 1.3% (effluent concentration of 0.3 ± 0.1 μg/L) to 4.4 ± 1.1% (effluent concentration of 4.4 ± 1.0 μg/L) during the first and the second period respectively, while N^4-Acetyl-SFX ranged from 0.2 ± 0.08% (effluent concentration of 0.02 ± 0.01 μg/L) to 0.1 ± 0.02% (effluent concentration of 0.06 ± 0.02 μg/L) during the first and the second period respectively.

4.4 Discussion

High degradation of SFX, up to 86%, was obtained during aerobic batch tests by the investigated enriched AOB and SFX degradation was clearly related to the nitrification rate with higher removals observed at higher SAORs. Similarly, Fernandez-Fontaina et al. (2012) observed that antibiotics and musk fragrances removal was found to be related to the nitrification performance in a NAS system, having the lowest biodegradation efficiency at the lowest specific nitrification rate value of 0.12 g NH_4^+-N/(g MLVSS d). Moreover, a linear relationship was found between the nitrifying activity and the removal of ibuprofen, erythromycin and roxithromycin in an aerobic conventional activated sludge system (Alvarino et al., 2014). On the other hand, previous findings reported kbiol values of less than 0.5 L/(g_{ss} d) for SFX, in batch tests carried out with CAS and membrane bioreactor sludge, suggesting its poor biodegradability (Joss et al., 2006; Suarez et al., 2010).

In the present study, SFX experienced a sharp decrease during the first half an hour in most tests that could be linked to adsorption onto the biomass. However, during the sorption control experiment that was executed, SFX did not show any remarkable removal during a period of four days. Similarly, Yang et al. (2011) conducted batch incubation experiments with activated sludge in order to study the sorption of sulfonamide antibiotics at an initial concentration of 100 μg/L at pH of 6.8. The study demonstrated that these compounds adsorb onto the activated sludge relatively quickly in the first 2 h, but this adsorption corresponded to a 6,5% of removal in the case of SFX. Moreover, they observed that when adsorption equilibrium was established biodegradation became the dominant mechanism.

A higher removal was obtained in the long term SBR-PN experiment with SFX being degraded up to 98% at both influent concentrations of 11.2 ± 0.8 and 101.7 ± 12.2 μg SFX/L. The SBR-PN was operating with a SAOR of 0.5 mg NH_4^+-N/(g MLVSS min). When

comparing this rate with the rates presented in Figure 4.2, a moderate removal of SFX would have been expected. However, the results demonstrated the high efficiency of a partial nitrification reactor in the removal of SFX without a previous period of adaptation, suggesting that the longer contact time of 24 h (compared to 6 h of the batch tests) played an important role on the removal of SFX. According to the literature, micropollutants having slow/intermediate kinetics such as some antibiotics experience less effective biodegradation at shorter HRTs (Fernandez-Fontaina et al., 2012). Moreover, another study stated that compounds with a half-life time less than WWTP HRT generally exhibit high removal efficiencies (García-Galán et al., 2011; Gros et al., 2010). Contrary to the findings of the present study, Müller et al. (2013) observed that SFX at an initial concentration of 10 mg/L remained unchanged, during a period of 14 days in 2L flask reactors operating under aerobic conditions with CAS, until adaptation of the biomass occurred. Moreover, SFX removal was found to be incomplete (36.5 ± 11.5%) in an SBR at 20^{o}C with CAS under nitrifying and denitrifying conditions, throughout an experimental period of two months, with no significant sign of enhancement over time, but with higher removals being obtained during the aerobic phases (Collado et al., 2013).

In the presence of ATU, nitrification was completely suppressed and this resulted in a null removal of SFX, providing an indication of the role of the AOB in SFX degradation. ATU is an inhibitor of copper-containing enzymes such as AMO, but also other monooxygenases that could possibly co-metabolize recalcitrant compounds. It was reported that any inhibition of AOB may negatively influence PhAC biodegradation in WWTPs due to the reduction in the growth rate of AOB (Sathyamoorthy et al., 2013). Likewise, the addition of ATU in an enriched nitrifier culture inhibited the nitrification completely and suppressed the removal of most selected pharmaceuticals (Tran et al., 2009). Similarly, the degradation rate constants of estrone, estradiol and ethinylestradiol were decreased significantly when ATU was added in a NAS system in the presence of ammonia-oxidizing bacteria Nitrosomonas europaea (Shi et al., 2004).

Some studies have investigated the expression of the AMO enzyme under ammonia starvation. Forrez et al. (2011), demonstrated that AOB cells starving for more than 2 months contained nearly twice as much AMO as actively growing cells, although they possessed lower ammonia-oxidizing activity. In other cases, AMO was even detected in Nitrosomonas after 1 year of NH_4^+ starvation, indicating a high resistance of AMO towards degradation

(Pinck et al., 2001). These findings could explain the removals around 28.4±7.1% that were observed in the present study in the absence of ammonia (T20-T21, Figure 4.2a), suggesting the potential of systems where there is an abundance of AOB towards the degradation of recalcitrant pollutants (even without a previous period of acclimation).

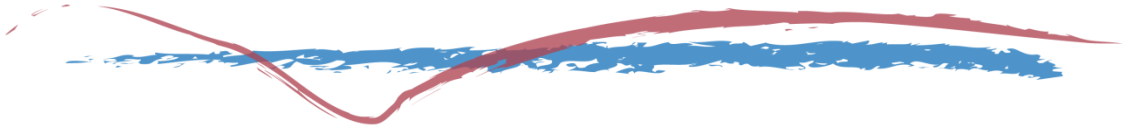
In this study, SFX TPs were found to represent up to 30% of the initial mass of SFX during the batch tests (in case that the latter was degraded) and up to 32% of the initial SFX concentration during the long term experiment**.** In both types of experiments**,** the main TP was 4-NO_2-SFX and SFX degradation occurred simultaneously and opposite to its formation. Desamino-SFX and N^4-Acetyl-SFX were detected in several experiments, even though at lower concentration. Similar results with 4-NO_2-SFX concentration developing opposite to that of SFX, were previously observed but in different conditions (aquifer material under denitrifying conditions, Barbieri et al., 2012). Finally, the short term experiments that were executed to explore the fate of the TPs in the absence of SFX, demonstrated that TPs could not be degraded. During the long term experiment it was observed that TPs were not cleaved back to SFX, since no accumulation of the latter was detected in the system. On the contrary, in batch tests involving aquifer material and under denitrifying conditions, 4-NO_2-SFX returned to the parent compound (SFX) when the concentration of NO_2^- dropped (Barbieri et al., 2012). This known reversible formation of nitro-derivatives, was not observed in this study taking into account that NO_2^- was accumulating into the system, in both short and long term experiments. Likewise, in contrast to the findings of the present study were Desamino-SFX was not found to be degradable, formation and its subsequent degradation was observed in anoxic water/sediment batch experiments with SFX and high NO_3^- concentration (Nödler et al., 2012). Moreover, N^4-Acetyl-SFX, was reduced in a concentration of 80-90% presumably via biodegradation, in an advanced wastewater reclamation plant (X. Yang et al., 2011). Same behavior was observed in batch biodegradation studies that were performed with CAS or with membrane bioreactor sludge, in which an exponential decrease of the concentration of N^4-Acetyl-SFX was observed over time (Joss et al., 2006). The contradictory results can be attributed to the different biomass and conditions applied.

In the present study the evaluated SFX TPs appeared to be stable, since non drop-back to the parent compound was observed, but they represent a significant percentage of the total removal. The results stress that only a comprehensive monitoring for metabolites or end products of mineralization can provide information about the real degree of biotransformation

of the parent compounds. In some cases, in fact, the concentrations of parent PhACs can even increase during the treatment process (Göbel et al., 2007; Lindqvist et al., 2005; Ternes, 1998), as specifically reported for sulfonamides (Joss et al., 2006; X. Yang et al., 2011). Moreover, Barbieri et al. (2012) reported that TPs such as 4-NO_2-SFX, may also be formed in nitrification and denitrification processes, leading to a wrong estimation of SFX removal efficiency, as this nitro derivative can transform back into the parent compounds.

Finally, TPs can exhibit similar or higher ecotoxicological effects than the parent compound. Majewsky et al. 2014 reported that 4-NO_2-SFX was found to inhibit bacterial growth to a clearly greater extent than the parent compound, SFX, whereas N^4-Acetyl-SFX retained less than 10% of the effect of SFX on growth and luminescence inhibition. Consequently, it is urgent to consider TPs formation as well in SFX degradation studies because they can contribute to the total antibacterial and ecotoxicological effect.

Chapter II



"Unraveling the potential of a combined nitritation-anammox biomass towards the biodegradation of pharmaceutically active compounds"

This Chapter was published as:

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5.1 Preliminary remarks

This study aimed to assess the degradation capability of combined N/A biomass towards five PhACs: IBU, SFX, MTP, CBZ and VEN. The biomass was withdrawn from the pilot-scale 400 L SBR-N/A (described in Section 3.2) treating digester supernatant. To investigate if degradation of the selected compounds (initial design concentration of 50 µg/L, each) will occur and to unravel which microbial groups are mainly involved in this activity, 24 h batch experiments were carried out under different conditions by selectively activating (presence of substrates and optimal conditions) or inhibiting (absence of substrates and/or presence of inhibitors) different microbial groups. The performed experiments aimed to promote a unique process in the mixed culture biomass: combined N/A, nitrification, anaerobic ammonium oxidation, denitrification, and finally aerobic oxidation of COD. Moreover, the formation of several major TPs was also investigated.

5.2 Materials and methods

5.2.1 Bioreactor set-up and operation

Biomass from a 400 L combined N/A pilot-scale reactor of both attached and suspended biomass treating digester supernatant was used for the batch experiments that were carried out in a fully automated 12 L reactor (described in Section 3.2). Prior to each experiment detailed in the following sections, 10 L of biomass were withdrawn and adequately prepared in order to meet the necessary initial conditions in terms of substrate concentrations and DO levels depending on each test. Samples for the analyses of nutrients, COD and PhACs, were taken along the experiment whereas total suspended solids (TSS) were measured at the end. More details on the set-up and operation of the reactor as well as on the chemical characterization are provided in Section 3.3.2 and 3.4.

5.2.2 Activity tests

Preliminary assays were performed in order to evaluate the activity of the bacterial communities present in the combined N/A reactor. The maximum activities of the three main autotrophic groups of microorganisms (anammox, AOB and NOB) as well as of the heterotrophic fraction present in the biomass were investigated prior to the execution of the

main batch experiments. The maximum anammox activity is defined as the nitrogen removal rate (sum of NH_4^+ and NO_2^-) in the absence of DO and under non-limiting concentrations of NH_4^+ and NO_2^-. The maximum AOB and NOB activities (under non-limiting DO concentration) are defined as the NH_4^+ removal rate through oxidation by AOB under non-limiting concentration of NH_4^+ and as the NO_3^- production rate through oxidation of NO_2^- by NOB under non-limiting concentration of NO_2^-, respectively. Finally, in order to evaluate the heterotrophic denitrifying activity, the NO_3^- removal rate in the absence of DO and under non-limiting concentrations of NO_3^- and acetate was studied.

The three activity assays were executed under different initial concentrations of NH_4^+ and NO_2^- and different duration taking into account the different yields of the microbial groups as well as the inhibitory effect of NO_2^- to anammox bacteria over specific concentrations as demonstrated in Lackner et al. (2014). NH_4^+ and NO_2^- were supplied as NH_4Cl and $NaNO_2$. In the case of the anammox activity test an initial concentration of ≥ 80 mg N/L for both NH_4^+ and NO_2^- was achieved in the system. For AOB and NOB activity test, NH_4^+ was supplied at 150 mg NH_4^+-N/L and NO_2^- at a low concentration of approximately 30 mg NO_2^--N/L, due to the immediate production by AOB that would ensure non-limiting conditions. Additionally, aerobic conditions were maintained (3 < DO < 3.5 mg/L) under active mixing. Finally, for the heterotrophs activity test $CH_3COONa.3H_2O$ was supplemented at an initial concentration of 900 mg COD/L whereas NO_3^- was already present in the system at a concentration of 1000 mg/L approximately (value in the effluent of the pilot-scale 400 L reactor).

The consumption and production rates were calculated by linear regression of off-line measurements. Four grab samples were taken from the bulk liquid phase with an interval of 45 minutes for anammox (3 h-test) and 30 minutes for AOB and NOB (2 h-test) activity tests. For the heterotrophic activity assay grab samples were collected at the beginning and after two, five and twenty-one hours (21 h-test). The different sampling times were selected based on previous results from activity tests.

The information gathered from the activity tests was used to design the batch experiments mainly in terms of substrate demand. All the experiments detailed in Section 5.2.3 were conducted after the activity tests here described.

5.2.3 Batch experiments

The aim of the experiments detailed in this section was to investigate the degradation capability of the combined N/A microbial community towards five PhACs (IBU, SFX, MTP, VEN and CBZ) and to unravel which microbial group was mostly responsible for this degradation. Moreover, the formation of several major TPs (1-hydroxyl-IBU, 2- hydroxyl - IBU and Carboxyl-IBU; 4-NO_2-SFX, desamino-SFX and N^4-acetyl-SFX; o-desmethyl-MTP, α-hydroxyl-MTP and MTP acid; o-desmethyl-VEN and n-desmethyl-VEN; 2- hydroxyl-CBZ and 10,11-epoxy-CBZ), was also investigated. Batch experiments were carried out under different conditions to selectively activate or inhibit different microbial groups: i) regular combined N/A operation, ii) aerobic (optimal for nitrifying bacteria-NB: AOB and NOB), iii) aerobic with ATU, iv) anoxic (optimal for anammox bacteria), v) aerobic with acetate (optimal for heterotrophic bacteria-HET) and vi) anoxic with acetate (optimal for denitrifying HET).

To address the degradation capability of the combined N/A microbial community, 24 h batch experiments were performed in a 12 L reactor at lab temperature (≈ 25 ^oC). During all experiments a mix of the five PhACs, at an initial concentration of 50 μg/L each, was spiked in the reactor while the substrates of AOB, anammox and HET (NH_4^+, NO_2^-, and COD, respectively) were also present in the system at different concentrations. NH_4^+ and NO_2^- were added using a pump, or spiked or depleted before the start of each experiment depending on the final aim. For the experiments i, ii, and iii, a stock of NH_4CL with a concentration of 4 g NH_4^+-N/L was prepared and added through a pump connected to a PLC and activated each time that NH_4^+ concentration was lower than 35 mg NH_4^+-N /L. In that way, the NH_4^+ concentration in the batch-scale reactor was maintained between 35 and 45 NH_4^+-N mg/L. During the experiment iv, a stock of NH_4CL (16 NH_4^+-N g/L) and a stock of $NaNO_2$ (16 NO_2^--N g/L) were prepared and spiked at the beginning of the test in order to achieve an initial concentration of approximately 45 NH_4^+-N mg/L and 55 NO_2^--N mg/L. These concentrations were selected based on the results obtained during the activity tests and the inhibitory effect of NO_2^- to anammox. The last two experiments were performed in the absence of substrates for AOB and anammox. On the other hand, NO_3^- was present in the system at a concentration of 1000 mg/L approximately. $CH_3COONa.3H_2O$ was added in the reactor in order to achieve an initial concentration of approximately 1300 mg COD/L, during the experiments v and vi. Since ATU is known to be degradable with time (Joss et al., 2011)

it was automatically added every 2 h (10 mL addition from a stock of 2 g/L) to assure complete inhibition of nitrification throughout the experiment. The different conditions applied in each experiment are presented in Table 5.1.

Samples were taken at the beginning of the test and after one, two, four, eight and twenty-four hours for chemical analysis of nutrients, PhACs and their TPs. TSS were measured at the end of each test.

Table 5.1: Description of the initial concentrations during the conducted experiments.

	DO	**Substrates**			**Inhibitor**		**PhACs**
Description		NH_4^+	NO_2^-	NO_3^-	ACE	ATU	
i) Combined N/A	0.05-0.1	35-45	<5	1000	---	---	50
ii) Aerobic-optimal NB	3-3.5	35-45	30	1000	---	---	50
iii) Aerobic-ATU	3-3.5	35-45	<5	1000	---	24	50
iv) Anoxic-optimal ANX*	0.0	35-45	55	1000	---	---	50
v) Aerobic-optimal HET	3-3.5	<1	<5	1000	1300	24	50
vi) Anoxic-optimal HET	0.0	<1	<5	1000	1300	---	50

Units: NH_4^+, NO_2^-, NO_3^- (mg N/L); ACE (mg COD/L); ATU (mg/L); DO (mg/L); PhACs (µg/L).
*In the tables and figures of the present thesis the acronym ANX stands for anammox bacteria.

5.2.4 Degradation rate constants calculations

Degradation rate constants were estimated. For each compound, zero (Eq. 5.1), first (Eq. 5.2) and second (Eq. 5.3) order equations were applied and the best fit of the kbiol constant for each compound and condition was selected.

$$Ct = Co - kbiol \cdot t \quad \text{(Eq. 5.1)}$$

$$lnCt = lnCo - kbiol \cdot t \quad \text{(Eq. 5.2)}$$

$$^1/_{Ct} = {}^1/_{Co} + kbiol \cdot t \quad \text{(Eq. 5.3)}$$

where C_t is the concentration of a particular compound at time t, C_o is the concentration at the start of the batch test (time 0 h), kbiol is the biodegradation rate constant, and t is the time.

5.3 Outcomes

5.3.1 Activity tests

The maximum activities of the three main autotrophic guilds present in the combined N/A biomass, as well as of the heterotrophic fraction, were investigated prior to the execution of the main batch experiments. Anammox bacteria displayed an activity of 267.5 mg (NH_4+NO_2)-N/L/d (Figure 5.1a). This value is much higher compared to the maximum anammox activity of 103 ± 18 and 138 ± 38 mg (NH_4+NO_2)-N/L/d (averaged over 5 months of operation) displayed in two reactors that were operated under combined N/A configuration on aerobically pre-treated municipal wastewater (Laureni et al., 2016). Likewise, AOB displayed an activity of 1936.1 mg NH_4^+-N/L/d (Figure 5.1b), which was much higher compared to the activity normally achieved in the pilot-scale 400 L reactor, where the DO was maintained at low concentrations. NOB activity was 878.4 mg NO_3^--N/L/d.

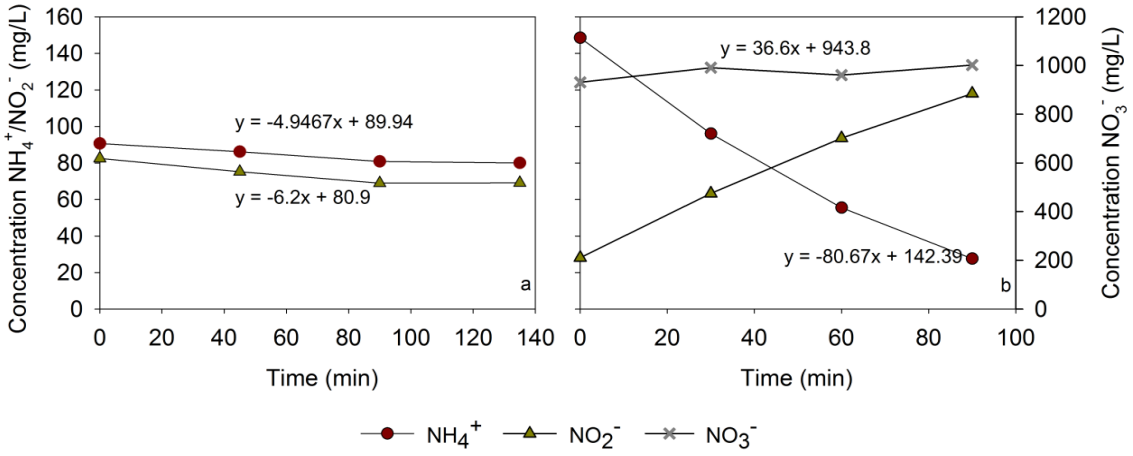


Figure 5.1: a) Anammox activity rate expressed as consumption of NH_4^+ and NO_2^-, b) AOB activity rate expressed as consumption of NH_4^+ at the simultaneous production of NO_2^- and NOB activity rate expressed as production of NO_3^-.

In the case of heterotrophs activity test, the activity was calculated at 123.5 mg NO_3^--N/L/d with a COD consumption of 635.7 mg COD/L/d, assuming that the uptake of COD from heterotrophic bacteria for reactions and growth equals to 3.5 mg COD/mg NO_2^--N and to 5.8 mg COD/mg NO_3^--N.

It is a general consensus that the presence of NOB inside a combined N/A reactor can cause process stability problems, especially because they can compete with AOB and anammox for

oxygen and for NO_2^-, respectively, changing the community dynamics. Nevertheless, in this case, the composition of the bacterial community of the 400 L combined N/A reactor was analyzed and results revealed that both AOB and anammox were present at higher abundances than NOB. The aerobic and anaerobic ammonia oxidizing community was dominated by AOB (*i.e.* genus *Nitrosomonas*) and anammox (*i.e. Candidatus* Brocadia) that represented 40% and 28.1%, respectively, of the carrier attached community. In turn, the suspended fraction (liquid phase) was dominated by AOB (82.8%). Finally, NOB (*i.e.* genus *Nitrospira*), represented a very small fraction with the relative abundance not exceeding 4.6% and 10.3% in the suspended and the carrier attached community respectively (Figure S14 in SM).

5.3.2 Batch experiments

The presence of the studied PhACs in the bulk liquid was measured prior to their addition during each experiment. CBZ and VEN were detected in all the cases at a concentration of 4.6 ± 0.45 μg/L and 1.4 ± 1.11 μg/L, respectively. IBU and MTP were detected only once at a concentration of 1.4 and 0.4 μg/L, respectively, whereas SFX was never detected in the matrix. A time course of the concentrations of the studied PhACs under aerobic conditions (exp. ii) is presented in Figure 5.2. Different degrees of removal as well as kinetics were obtained for the different compounds. IBU experienced a rapid decrease during the first two hours with a lower decrease rate in the rest of the experiment and exhibited the highest removal (81%) followed by SFX (70%) and MTP (37%), whereas CBZ and VEN demonstrated a recalcitrant behavior with removals being lower than 13%.

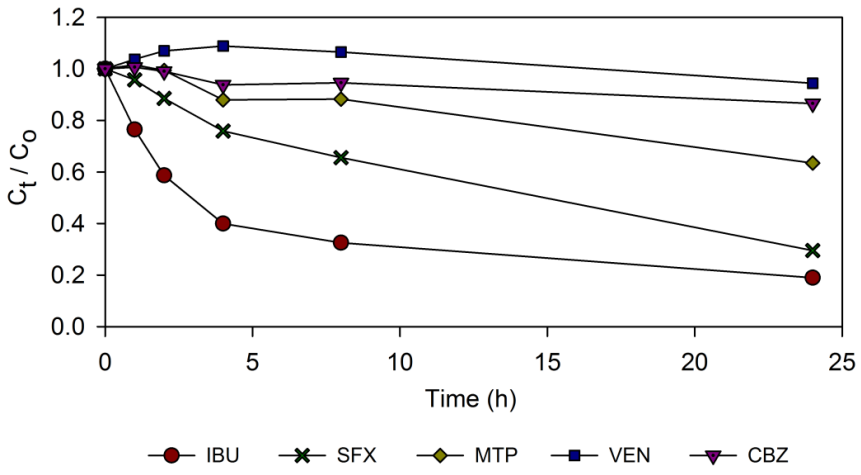


Figure 5.2: Changes in normalized concentrations of the studied PhACs during experiment ii.

Likewise, IBU was the most removed compound, under all the different experimental conditions, followed by SFX and MTP. On the other hand, CBZ and VEN exhibited the lowest removals (Figure 5.3).

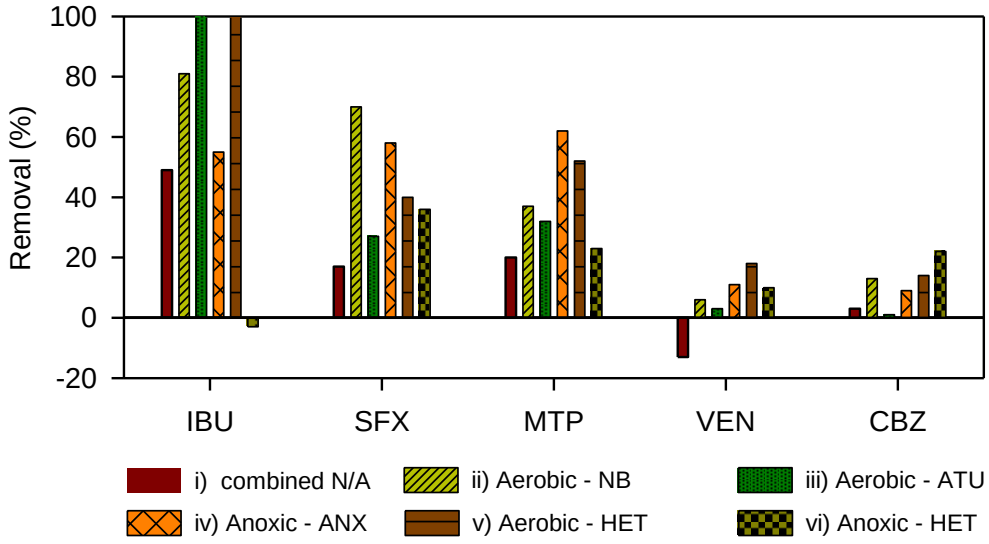


Figure 5.3: Removal of the studied PhACs under the different experimental conditions.

More in detail, IBU exhibited from moderate to high removals (49-100%) except in the case of heterotrophic denitrification (exp. vi). Notably, the highest performance (81-100%) was displayed when aerobic conditions were applied. Regarding SFX, 70% and 58% removal was detected when the activities of AOB and anammox were studied independently (exp. ii and iv, respectively). Surprisingly, when both populations were active (exp. i) the lowest removal was detected, reaching values around 20%. In the presence of ATU (exp. iii), nitrification was completely suppressed, fact that resulted in a decrease in SFX elimination (only 27% removed). The results indicate the role of AOB but also the partial contribution of other microbial groups present in the sludge. Indeed, the contribution of heterotrophic bacteria cannot be disregarded since up to 40% elimination was observed during a typical conventional activated sludge (CAS) operation (v), as well as during heterotrophic denitrification (vi). MTP was moderately removed (up to 62%) and the highest performance was achieved under anoxic conditions favoring anammox bacteria (exp. iv), whereas 37% of removal was observed when AOB were favored (exp. ii). With both populations active (exp. i), as previously presented for SFX, only a 20% of MTP was degraded. Additionally, the heterotrophic fraction contributed up to 23 and 52% during the experiments vi and v, respectively. Finally, CBZ and VEN displayed a quite recalcitrant behavior with removals

being lower than 10% under most of the conditions tested. Therefore, no difference in the degradation capabilities of the studied microbial groups was considered for these compounds. It should be noted that the possible contribution of NOB towards the degradation of the studied compounds cannot be discarded although they represented a small fraction of the combined N/A community in comparison to that of AOB and anammox (as indicated in Section 5.3.1).

The removal achieved in the presence of ATU, related to AMO and AOB inhibition, was lower (except for IBU) compared to the one achieved when AOB were active, indicating a partial contribution of this microbial group.

The formation of few major TPs was evaluated in parallel to the degradation of the parent compounds in all the batch experiments. In the case of SFX, MTP, CBZ and VEN, the studied TPs (i.e.: 4-NO_2-SFX, desamino-SFX and N^4-acetyl-SFX; o-desmethyl-MTP, α-hydroxyl-MTP and MTP acid; 2-hydroxyl-CBZ and 10,11-epoxy-CBZ; o-desmethyl-VEN and n-desmethyl-VEN) were always below limit of detection or below limit of quantification (Table 3.2). On the other hand, in the case of IBU, 1-hydroxyl-IBU was formed up to 2.07 μg/L with an average of 0.66 ± 0.59 μg/L, 2-hydroxyl-IBU was formed up to 1.97 μg/L with an average of 0.8 ± 0.54 μg/L, whereas carboxyl-IBU was always below limit of detection (Table 3.2). Although different behaviors were observed, it seems that the microbial communities thriving in a combined N/A reactor have the capacity of eliminating these two IBU TPs (i.e. 1-hydroxyl-IBU and 2-hydroxyl-IBU), except in the case of AOB (exp. ii). Time course data of IBU and its detected TPs for all the applied experimental conditions is provided in SM (Figure S15).

Kbiol constants were estimated from the batch concentration time series. In the case of CBZ and VEN very slow degradation rates and low removals (≤ 10%) were observed for the majority of the experiments and their behavior is not being further discussed. Zero, first and second order kinetics were calculated for the rest of the compounds (refer to section 2.5) under all the experiments except for iv (optimal anammox), discussed later. The degradation of SFX obeyed first-order reaction kinetics, whereas of MTP second-order reactions kinetics. In most cases the experimental data were fitted satisfactorily and achieved high R^2 values (Table 5.2).

Table 5.2: Degradation rate constants of IBU, SFX and MTP under the conducted experiments

	i)N/A	R^2	ii)aerobic -AOB	R^2	iii)aerobic - ATU	R^2	v)aerobic -HET	R^2	vi)anoxic -HET	R^2
IBU	$0.63^{(1)}$	0.98	$0.19^{(2)}$	0.96	$6.84^{(1)}$	1.00	$7.17^{(1)}$	0.99	n.a	--
SFX	n.a	--	$1.21^{(1)}$	1.00	$0.28^{(1)}$	0.64	$0.54^{(1)}$	0.98	$0.50^{(1)}$	0.88
MTP	$0.01^{(2)}$	0.96	$0.02^{(2)}$	0.97	$0.01^{(2)}$	0.74	$0.04^{(2)}$	0.99	$0.02^{(2)}$	0.95

$^{(1)}$: pseudo-first order (1/d) and $^{(2)}$: pseudo-second order degradation ($\mu g/L^{-1}\ d^{-1}$)
n.a.: not available values since the regression line could not approximate the real data points

Different degradation rates were obtained for IBU, SFX, MTP (Figure 5.4a, b and c, respectively) highlighting the influence of the activated microbial groups and applied conditions. IBU experienced a rapid decrease during the experiments ii, iii, iv and v, whereas in the case of combined N/A (exp. i) and heterotrophic denitrification (exp. vi) it displayed a slower degradation rate (Figure 5.4a). Similar degradation rates were obtained under the different conditions tested for SFX with the exception of the anammox experiment (exp. iv) where a faster degradation was obtained during the first 4 h (Figure 5.4b). Likewise, MTP exhibited slow degradation rates and its removal was relatively low during the first 8 h, except during the anammox experiment (exp. iv) where it experienced a very sharp decrease and within 2 h the final removal was nearly achieved (Figure 5.4c). Notably, IBU, SFX and MTP appeared to have a similar behavior during experiment iv. The rapid decrease during the first 2 to 4 h that was followed by an almost null removal during the rest of the experiment, suggests an inhibition of anammox bacteria although neither DO excess, nor substrate limitation or excess above the non-inhibitory level was detected (NH_4^+ and NO_2^- concentration did not, in fact, exceed 66 mg NH_4^+-N/L and 60 mg NO_2^--N/L respectively, values which were actually lower compared to those measured in the activity tests).

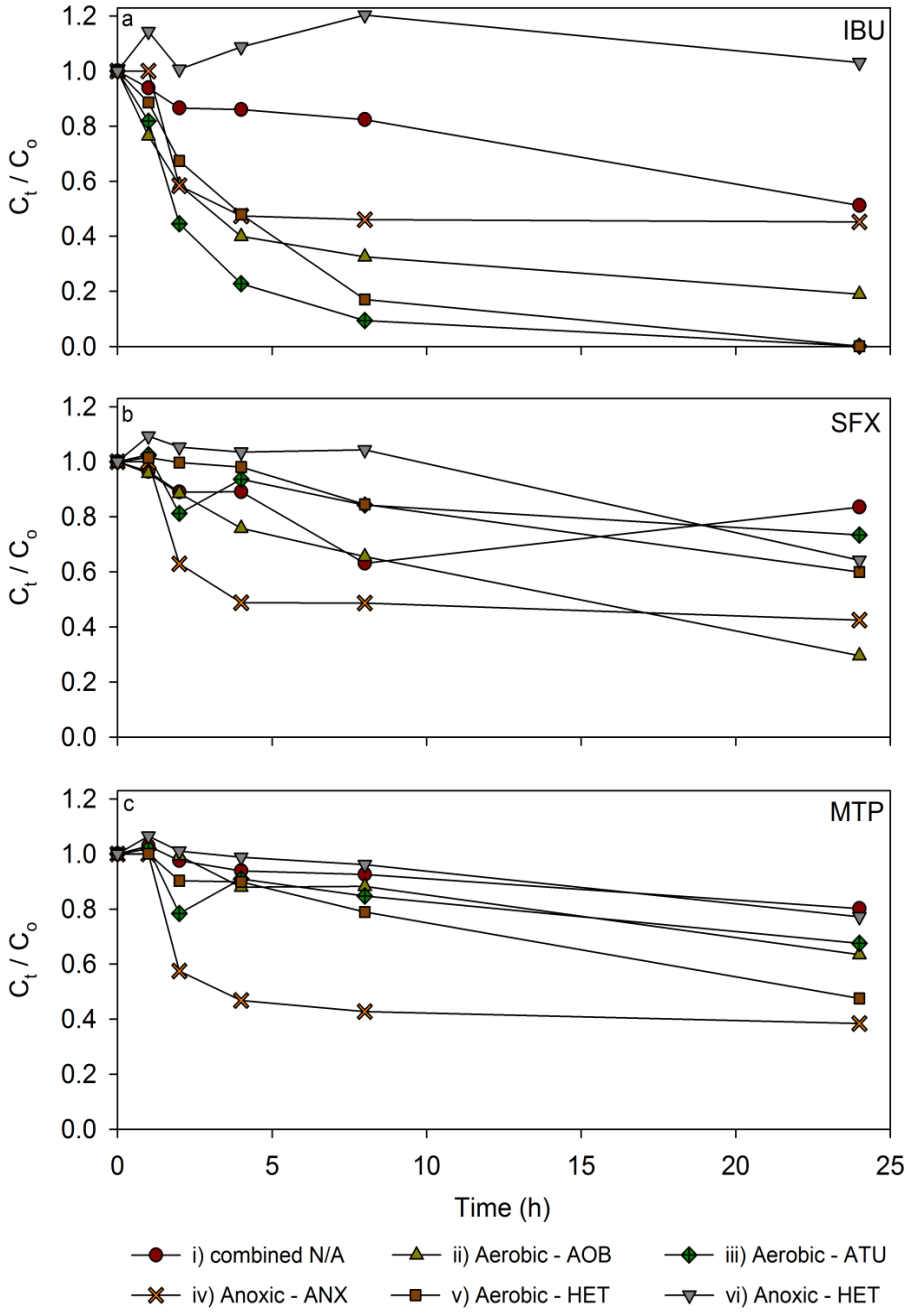


Figure 5.4: Normalized concentrations of IBU (a), SFX (b) and MTP (c) under all the different experimental conditions tested.

The PhACs removals, compared with literature data obtained with similar system and microbial populations are presented in Table 5.3.

Table 5.3: Comparison of the removals of the studied PhACs with the removals obtained in similar systems and biomass found in literature.

Conditions	**Removal (%)**									
PhACs	**IBU**	**Ref.**	**SFX**	**Ref.**	**MTP**	**Ref.**	**CBZ**	**Ref.**	**VEN**	**Ref.**
i) Combined N/A	**49**	98±1[a]	**17**	57±2[a], ≈ 50 SFX+N[4]-Acetyl-SFX[f]	**20**	<5[f]	**3**	7±1[a], negative [f]	**-13**	<10[f]
ii) Aerobic-optimal AOB	**81**	>96[e], 95±4[g], ≈ 100[h], 16[c]	**70**	22±5[g], 44[e]	**37**	67[c]	**13**	≈ 20[e], 6±12[g], <40[h], -[c]	**6**	n.a.
iii) Aerobic-ATU	**100**	51[e], ≈75[h]	**27**	93[e]	**32**	n.a	**1**	<10[e], ≈12[h]	**3**	n.a.
iv) Anoxic-optimal ANX	**55**	77[c]	**58**	n.a.	**62**	≈ -23[c]	**9**	-[c]	**11**	n.a.
v) Aerobic-optimal HET	**100**	51[e], ≈75[**h] and 94[*h], >95[b]	**40**	<45[b], ≈ 75 SFX+N[4]-Acetyl-SFX[d], 93[e]	**52**	≈ 25[d]	**14**	-25[d], 0[b], <10[e,*h], ≈ 25[**h]	**18**	<10[d]
vi) Anoxic-optimal HET	**-3**	37±26[g]	**36**	>80 SFX+N[4]-Acetyl-SFX[d]	**23**	≈40[d]	**22**	≈-25[d], 1±10[g]	**10**	≈40[d]

[a] Alvarino et al. (2015); [b] Alvarino et al. (2014); [c] de Graaff et al. (2011); [d] Falås et al. (2016); [e] Fernandez-Fontaina et al. (2016); [f] Laureni et al. (2016); [g] Suarez et al. (2010); [h] Tran et al. (2009) : *without and **with addition of external carbon. n.a. stands for non available data.
To be noted that for the experiments v and iv, CAS biomass was considered in some cases (b, d and h*) due to the lack -in literature- of studies with the exact same configuration and biomass.

5.4 Discussion

It is known that the main removal mechanisms of micropollutants during biological treatment are biodegradation/biotransformation and sorption, while volatilization occurs to a minor degree (Verlicchi et al., 2012). Indeed, the PhACs selected for this study are almost non-volatile (Fernandez-Fontaina et al., 2014; SRC, 2011; Suarez et al., 2010). Regarding sorption, the Kd coefficient has been proposed as a relative accurate indicator (Joss et al., 2005; Ternes et al., 2004) since it takes into account the two main sorption mechanisms: absorption (characterized by the K_{ow} value) and adsorption (characterized by the dissociation constant, pKa) (Schröder et al., 2016). For SFX, MTP and CBZ median Kd were calculated by Falås et al. (2016) at 0.04, 0.15 and 0.013 L/gSS respectively. In the case of IBU Kd was estimated at 0.007 ± 0.002 L/gSS (Ternes et al., 2004). Those values are lower than 0.3 L/g, indicating that sorption onto the secondary sludge is not relevant, as reported by Joss et al. (2005). As for venlafaxine, it has been recently reported that its adsorption was lower than some compounds here evaluated (Lucas et al., 2018). Therefore, the studied PhACs are typically low sorbing (Kd ≤ 0.15 L/gSS) and the removal here presented and discussed could mainly be attributed to (bio)degradation.

5.4.1 Factors affecting the removal of micropollutants

With the aim of investigating the contribution of different microbial groups, distinct conditions were studied in order to simulate different treatment scenarios that occur at full-scale. The experiments carried out were conducted to activate a unique process in the mixed culture biomass: nitrification, anaerobic ammonium oxidation, denitrification, aerobic oxidation of COD and finally a combined N/A. It is worth to mention that the use of ATU as inhibitor was solely performed to unravel the role of AOBs and not to mimic any actual scenario.

In the case of CBZ and VEN, little degradation was observed (≤ 10% in most cases) independently of the conditions tested. The results are in accordance with previous studies where a quite recalcitrant character of these two compounds was demonstrated (Alvarino et al., 2014; Jelic et al., 2015; Joss et al., 2006; Laureni et al., 2016; Luo et al., 2014; Verlicchi et al., 2012). Likewise, Falås et al. (2016) stated that the spectrum of organic micropollutants susceptible to biological degradation at WWTPs can be broadened by combining different

aerobic and anaerobic treatment conditions, but even so, some micropollutants must be considered stable. Regarding the rest of the studied compounds (IBU, SFX and MTP), their removal varied depending on different factors, discussed below.

Redox conditions

IBU exhibited from moderate to high removals (49-100%) except in the case of the heterotrophic denitrification (exp. vi) where a practically null removal was detected. Similarly, previous studies showed that although IBU is considered to be highly biodegradable, much lower removals (37 ± 26% and 15%) were achieved during anoxic batch experiments executed with conventional activated sludge (CAS) performing denitrification (Suarez et al., 2010; Zwiener et al., 2000, respectively). It is therefore deduced that the biodegradability of a compound is not the only driving force, and that redox conditions in combination with the active microbial group involved contribute in a significant way. The dependency of biodegradation on redox conditions was also demonstrated by Sgroi et al. (2018). The authors suggested that the occurrence of a large redox range in a partially saturated constructed wetland may enable complex microbial interactions under aerobic and anaerobic microenvironments or promote the presence of certain microbial communities that can influence the removal of some contaminants. This observation accords well also with the finding of Falås et al. (2016), where it was demonstrated that different redox conditions can improve the degradation of certain micropollutants. Regarding SFX, relatively higher removals (up to 18%) were achieved during anoxic conditions (exp. iv and vi), compared to the removals obtained during oxic conditions (exp. i, iii and v) that could -up to a point- be attributed to the fact that SFX is strongly dependent on the reduction potential with its removal being higher under denitrifying conditions, as previously stated (Banzhaf et al., 2012). These observations are consistent with those of Sgroi et al.,(2018), where it was reported that although aerobic conditions are generally found to be more favorable for the removal of micropollutants, in the case of SFX non aerobic environments led to improved elimination. On the other hand though, a high removal of 70% was observed under NB optimal conditions (exp. ii). In the latter case it seems that the removal of SFX was not triggered by the redox conditions but rather by the microbial group that was favored, or by the nitrification rate as discussed later on.

Nitrification rate and ATU

An interesting result was obtained for all the compounds during the typical operation of combined N/A (exp. i) when both AOB and anammox were active. The removal was always lower in comparison to the removals achieved independently by the aerobic and anoxic populations and under optimal conditions (exp ii and iv, respectively). In the case of experiment ii (optimal AOB), a possible explanation could be the high nitrification rate (5.28 mg NH_4^+-N/min) in comparison to the low nitrification rate (0.39 mg NH_4^+-N/min) that was achieved during the experiment i (combined N/A). Previous studies have indeed shown a link between the nitrification rate and the degradation of some compounds, with higher removals being obtained under higher nitrification rates (Alvarino et al., 2014; Fernandez-Fontaina et al., 2012; Kassotaki et al., 2016). In the case of experiment iv (optimal anammox), the nitrification rate achieved was lower than the one during the combined N/A operation (0.25 mg NH_4^+-N/min), but notably IBU, SFX and MTP exhibited relatively high removals (55%, 58% and 62% respectively) when compared to those achieved during the combined N/A operation (49%, 17% and 20% respectively). The results suggest the degradation capability of the anammox bacteria when operating under optimal conditions.

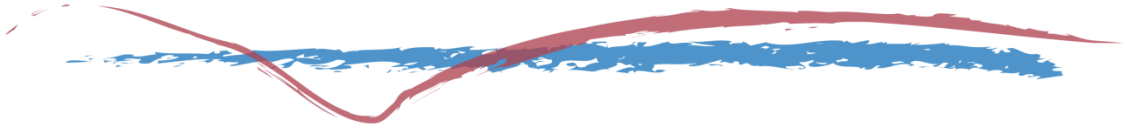
The addition of the inhibitor ATU affected differently the studied compounds. In the case of MTP, the similar removal observed in the presence or absence of ATU (32% and 37% respectively) suggests only a small contribution of AOB towards its degradation. On the other hand, an important contribution was found for SFX where in the presence of ATU, its removal dropped from 70 to 27%. Similar results were previously obtained during batch experiments with AOB and SFX (Kassotaki et al., 2016). Interestingly, no ATU inhibition on IBU degradation was observed indicating that degradation was carried out by ATU-insensitive enzymes as formerly reported (Men et al., 2017; Tran et al., 2009).

Specific biomass capabilities

Regarding the different factors evaluated in the present study, it could be concluded that each biomass has unique metabolic capabilities that affect the removal efficiencies of micropollutants. The latter was demonstrated, by the fact that IBU exhibited a null removal under anoxic conditions aiming to heterotrophic denitrification. Moreover, contrary to the findings of de Graaff et al. (2011) where no removal was observed in an anammox reactor, here, MTP displayed the highest degradation during anoxic conditions favoring anammox

bacteria (exp. iv). Additionally, MTP exhibited 20% removal during the combined N/A operation (exp. i), whereas an almost negligible removal was found in two other combined N/A systems (i.e. a moving bed bioreactor run only with carriers and a hybrid MBBR run with carriers and flocculent biomass) (Laureni et al., 2016), indicating that the growth of diverse microbial populations can lead to different degradation capabilities.

Chapter III



"Comparative assessment of endocrine disrupting compounds removal in heterotrophic and enriched nitrifying biomass"

This Chapter was submitted as:

Elissavet Kassotaki, Maite Pijuan, Ignasi Rodriguez-Roda, Gianluigi Buttiglieri. 2018. Comparative assessment of endocrine disrupting compounds removal in heterotrophic and enriched nitrifying biomass.

6.1 Preliminary remarks

In the present study, autotrophic and heterotrophic types of biomass were compared in terms of efficiency in the removal of five EDCs of concern: E1, E2, E3, EE2 and BPA. Batch experiments were carried out with enriched NAS and enriched AOB sludge cultivated at lab-scale, as well as with CAS from a full-scale wastewater treatment plant. In order to corroborate the role of nitrifying bacteria and/or to disclose the role of heterotrophic bacteria in the removal of EDCs (initial design concentration of 15 μg/L, each), series of 2 h batch experiments were carried out. The aim was to: i) explore the effect of increasing nitrification rates on the elimination of the selected EDCs in the enriched NAS and AOB biomass; ii) investigate the contribution of the heterotrophic bacteria and the small autotrophic fraction present in CAS in the presence of different combination of substrates; and iii) evaluate the role of AMO enzyme.

6.2 Materials and methods

6.2.1 Bioreactor set-up and operation

Two fully automated SBRs of 8L were inoculated with activated sludge from a domestic WWTP located in Girona (Spain). The reactors were operated differently in order to promote full nitrification and partial nitrification and to develop an enriched NAS and AOB culture, respectively. More details regarding the set-up and operation of the reactors are provided in Section 3.1.1 and 3.1.2.

The batch experiments detailed in Chapter III were performed after more than 1 year of reactors' operation. In that sense, complete adaptation of the biomass of both reactors had been obtained. A stable full nitrification (95% of NH_4^+ converted to NO_3^- and no NO_2^- detected in the effluent) and partial nitrification (more than 80% of the total microbial community belonging to the AOB group and 95% of NH_4^+ converted to NO_2^- with no NO_3^- detected in the effluent) had been established in the SBR-FN and SBR-PN, respectively.

Finally, in the case of CAS, fresh biomass was taken the same day of the experiments from the aerobic basin of the WWTP of Girona (Spain).

6.2.2 Batch experiments with NAS, AOB and CAS

In order to unravel which microbial groups (among NAS, AOB and CAS) are mainly involved in the degradation of E1, E2, E3, EE2 and BPA, series of 2 h batch experiments were carried out to: i) explore the effect of increasing nitrification rates on the elimination of the selected EDCs in the enriched NAS and AOB biomass; ii) investigate the contribution of the heterotrophic bacteria and the small autotrophic fraction present in CAS in the presence of different combination of substrates; and iii) evaluate the role of AMO enzyme.

Prior to the start of the experiments, a mix of E1, E2, E3, EE2 and BPA at an initial design concentration of 15 μg/L each, was spiked in each Schott bottle. The intermediate stock of the EDCs' mixture was allowed to evaporate under a gentle stream of N_2 for approximately 15 min, to minimize the potential inhibitory effects of methanol solvent on the biomass and to avoid the addition of an extra carbon source to the medium. The stock solution was then reconstituted in water under sonication before its final addition in the medium.

The substrates of autotrophic and heterotrophic bacteria (NH_4^+ and acetate, respectively) were also added in the system at different concentrations or combinations depending on the final aim of each test (Table 6.1). As regards to NAS and AOB biomass, stock solutions of NH_4HCO_3 at various concentrations were used in order to achieve different initial concentrations of NH_4^+ that resulted in different SAORs. Specifically, for both NAS and AOB biomass, duplicates were carried out for each of the selected NH_4^+ design concentrations (i.e. 100, 50 and 30 mg NH_4^+-N/L) (experiments T1-T6 and T11-T16 for NAS and AOB, respectively). Moreover, duplicates were executed in the absence of NH_4^+ (experiments T7-T8 and T17-T18 for NAS and AOB, respectively, Table 6.1).

On the other hand, in the case of CAS biomass, experiments were executed under: i) complete absence of substrates (experiments T21-T22) or in the presence of substrates for ii) only the autotrophic fraction of the microbial community (NH_4^+) (experiments T23-T24), iii) only the heterotrophic fraction (acetate) (experiments T25-T26) or iv) both fractions (NH_4^+ and acetate) (experiments T27-T28) (Table 6.1). As regards to acetate, a stock solution of CH_3COONa was used as a substrate in order to achieve an initial design concentration of 100 mg/L.

Finally, experiments were executed for the three types of biomass in the presence of ATU, at 20 mg/L, to suppress AMO and consequently NH_4^+ oxidation (experiments T9-T10, T19-T20 and T29 for NAS, AOB and CAS, respectively).

Samples from the bulk liquid were taken prior to the addition of substrates and EDCs, just after their addition and from that point onwards at 30 min intervals (i.e. time start-control and 0, 30, 60, 90 and 120 min) for chemical analysis of EDCs, N species and acetate. MLSS and volatile MLSS (MLVSS) were measured at the beginning of each test.

Finally, abiotic experiments (without biomass) were performed confirming negligible abiotic removal (difference between initial and final value < 5%), thus ruling out chemical transformations as a relevant mechanism for the selected compounds.

Table 6.1: Description and initial design concentrations in the conducted experiments

	Description	Code	NH_4^+-N (mg/L)	ACE (mg/L)	EDCs (µg/L)	ATU (mg/L)
NAS	**Different NH_4^+ scenarios**	T1	100	--	15	--
		T2	100	--	15	--
		T3	50	--	15	--
		T4	50	--	15	--
		T5	30	--	15	--
		T6	30	--	15	--
		T7	--	--	15	--
		T8	--	--	15	--
	NAS + ATU	T9	50	--	15	20
		T10	50	--	15	20
AOB	**Different NH_4^+ scenarios**	T11	100	--	15	--
		T12	100	--	15	--
		T13	50	--	15	--
		T14	50	--	15	--
		T15	30	--	15	--
		T16	30	--	15	--
		T17	--	--	15	--
		T18	--	--	15	--
	AOB + ATU	T19	50	--	15	20
		T20	50	--	15	20
HET	**Only EDCs**	T21	--	--	15	--
		T22	--	--	15	--
	NH_4^+	T23	50	--	15	--

Description	Code	NH_4^+-N (mg/L)	ACE (mg/L)	EDCs (μg/L)	ATU (mg/L)
	T24	50	--	15	--
ACE	T25	--	100	15	--
	T26	--	100	15	--
NH_4^+ + ACE	T27	50	100	15	--
	T28	50	100	15	--
NH_4^+ + ACE + ATU	T29	50	100	15	20

6.2.3 Additional experiments with CAS

Further experiments were carried out with CAS in order to thoroughly investigate the fate of the studied EDCs.

Independent experiments were conducted in duplicates spiking CAS biomass with solely E2 (at an initial design concentration of 15 μg/L) to unravel the correlation between E2 and E1. Moreover, to assess possible losses of E1, E2, E3, EE2 and BPA due to mechanisms other than biodegradation, sorption control experiments were performed with heat-inactivated (autoclaved) biomass (20 minutes at 121°C and pressure).

In the case of NAS and AOB their low performance in terms of EDCs' removal did not allow for further investigation.

6.3 Outcomes and Discussion

6.3.1 EDCs' elimination at different SAORs

The experimental NH_4^+ concentrations varied in comparison to the designed ones and differences in the obtained SAORs were detected between duplicates (Table S3 in SM), thus each experiment was treated individually.

The results of the experiments performed with enriched NAS revealed that EDCs' removal was not correlated with the SAORs given the similar removals exhibited in all cases (Figure 6.1a). Similar results were also obtained for the enriched AOB community (Figure 6.1b), suggesting that the increasing activity of AOB, measured as nitrification rate, did not enhance the removal of the selected compounds in our tests and conditions.

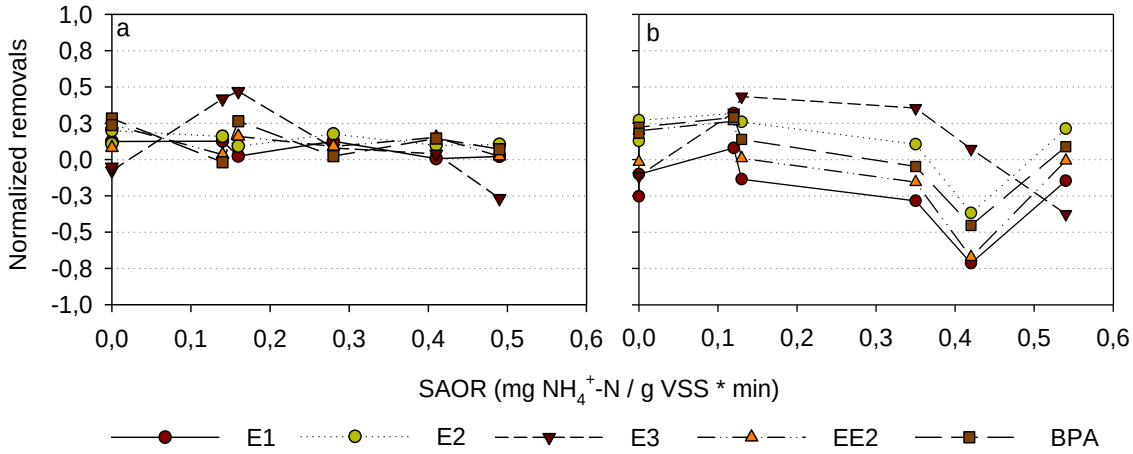


Figure 6.1: EDCs' removal as a function of SAOR during batch experiments with enriched a) NAS and b) AOB biomass. Results are expressed as normalized removals (C_o-C_{end}/C_o).

On the contrary, previous studies have indicated the positive correlation of nitrification rate and EDCs removal. Dytczak et al. (2008) demonstrated that higher removal rates of E2 and EE2 were associated with higher nitrification rates. Similar results were obtained by Ren et al. (2007), who indicated that the biodegradation rates of E1, E2 and EE2 were dependent on the activity of AOB, with better nitrification rates leading to faster degradation. Additionally, Yi and Harper (2007) demonstrated that EE2 biotransformation rate increased from 1.1 to 4.1 µmol EE2/g VSS/h, with an increase of NH_3 biotransformation rate from 0.3 to 3.1 mmol NH_3/g VSS, result that strongly shows a linear link between nitrification and EE2 removal in enriched nitrifying cultures.

Notwithstanding the here studied nitrification rates were found to have no influence on EDCs, a plateau removal would at least have been expected since the efficiency of nitrifying systems has previously been reported (De Gusseme et al., 2009; Shi et al., 2004; Vader et al., 2000). In a point of fact, Chen et al. (2013) investigated the removal of E1 and EE2, in three aerobic MBRs, and a linear correlation was exhibited between the different nitrification rates and the number of AOB. Nonetheless, EDCs' removal rates were not linearly correlated with either the abundance of AOB or the nitrification rates (in line with our results) but even so, average removals of 69.4% and 52.4% were attained for E1 and EE2 respectively. Contrariwise, here, both enriched NAS and AOB displayed a low degradation capability and very low removals were obtained under all the investigated scenarios (Figure 6.2a and b, respectively).

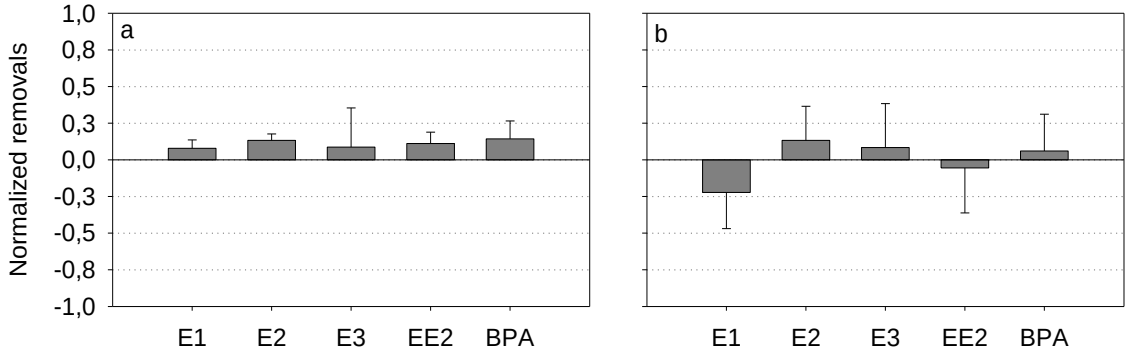


Figure 6.2: Average of normalized removals (C_o-C_{end}/C_o) for each EDC under all the NH_4^+ scenarios investigated in the enriched a) NAS and b) AOB, biomass. The error bars represent standard deviation.

The removals exhibited were quite low (≤ 0.14) when compared to those achieved with NAS in the literature. Indeed, up to 100 % removal of the studied estrogens (at varying initial concentrations) was achieved in different lab scale studies (in reactors or batch configurations) under the use of different inoculums (Amin et al., 2017; De Gusseme et al., 2009; Shi et al., 2004; Yi and Harper, 2007). Likewise, high efficiencies have been reported mainly in the nitrifying compartments of WWTPs. Andersen et al. (2003) stated that E1 and E2 were degraded biologically up to 98% in the denitrifying and aerated nitrifying tanks, whereas EE2 was reduced by more than 90% but only under nitrification. In addition, McAdam et al. (2010) demonstrated biodegradation efficiencies of 51%, 80% and 91% for carbonaceous, nitrification/denitrification and nitrification activated sludge process, respectively.

Many factors such as compound biodegradability, biomass adaptation, substrate competition, HRT and compound concentration, seem to be critical and may explain the low performance of the enriched NAS and AOB biomass of the present study.

According to literature E2 and E1 are readily biodegradable, whereas E3, EE2 and BPA are more persistent, behavior which affects their biodegradation rates and final percentage of removal (Hashimoto and Murakami, 2009; Shi et al., 2004). The order of degradation rate constants was found to be: E2 > E3 > E1 > EE2 in batch experiments with NAS and under different substrates and organic loading rates (Ren et al., 2007), whereas slightly different results were obtained in other studies with NAS (Amin et al., 2017; Shi et al., 2004). As regards to BPA, it was found to be more readily biodegradable as compared to EE2 in batch experiments with nitrifiers (Bautista-patacsil et al., 2014). In the present study, the selected

EDCs exhibited the same low removals in both NAS and AOB batch experiments, suggesting the poor capability of nitrifiers and/or the influence of other factors, as explained below.

The lack of biomass adaptation could possibly lead to the here detected low performance. Indeed, a lag phase between biomass exposure to estrogens and onset of significant degradation was observed in other studies, suggesting the need of acclimation period. Both EE2 and BPA were almost completely degraded by nitrifying sludge, however, considerable removal was only detected after > 6 h of experiment (Kim et al., 2007; Vader et al., 2000). Additionally, batch tests with commercially available nitrifier enrichment culture (NEC) demonstrated that the maximum EE2 removal rate was achieved after 72 h, when all NH_4^+ had been oxidized into NO_3^-, indicating potential competition effect between NH_4^+ and EE2 for (enzymatic) removal by the NEC (De Gusseme et al., 2009).

At the same time, both SRT and HRT have appeared to be especially important parameters in removing EDCs from secondary treatment systems (Amin et al., 2017; Siegrist et al., 2005; Ting and Praveena, 2017). The higher biodegradation efficiencies under longer SRTs have been associated to the development of a more diverse microbial community, including the growth of nitrifying bacteria (Clara et al., 2005; Sathyamoorthy et al., 2013). On the other hand, in the case of HRT, prolonged contact times can be quite relevant especially for compounds having slow/intermediate kinetics. Long contact time of 96 h led to almost 100% removal of E1, E2, E3 and EE2 (at 1 mg/L) in batch experiments with NAS, while other studies with EE2 at contact times of 150 and 240 h, led to ≥ 95% removal at both concentrations of 50 μg/L and 750 μg/L, respectively (De Gusseme et al., 2009; Vader et al., 2000). Similarly, high degradation (> 80%) of BPA at initial concentrations of 10 mg/L and 100 mg/L were achieved by enriched NAS during batch experiments of 48 h and 7 d, respectively (Kim et al., 2007). The negligible elimination detected in the present study might be attributed to the very short contact time (2 h). However, most WWTPs designed for municipal wastewater have a typical HRT of approximately 4-12 h (Liu et al., 2015) and, hence, the contact time here selected is more relevant compared to those mentioned previously.

Moreover, to be noted that the concentrations of the above referred studies are often way beyond (up to approximately 10^5 times) those typically found in WWTPs and in the environment. Normally, WWTPs' influent concentrations of E1, E2, E3, EE2 and BPA range from below detection to 670 ng/L for E1, 150 ng/L for E2, 660 ng/L for E3, 70 ng/L for EE2

and 1μg/L for BPA (Gaulke et al., 2008; Hamid and Eskicioglu, 2012; Luo et al., 2014). It can thus be speculated that the rates of consumption could be affected by these extremely high concentrations: at the mg/L level any compound could be considered a macro rather than a micro-pollutant and could even be used as a primary substrate by bacteria.

Although in the above referred studies nitrification has been cited as the driving force in EDCs' removal with a marginal role of the heterotrophic bacteria, many other studies align well with our findings. A recent review suggested that although prolonged SRTs can improve a WWTP's natural estrogen removal performance, slow-growing nitrifying bacteria do not seem to play a decisive role in this improvement (Liu et al., 2015). Indeed, both Bagnall et al. (2012) and McAdam et al. (2010) declared that although the highest removals of EDCs are only achieved during nitrification, it is the process conditions that support nitrification, for example extended SRT, high mixed liquor concentrations and contact time, as opposed to nitrification activity (and the role of AMO) per se, which promote estrogen biodegradation. Their conclusions are in line with the findings of Petrie et al. (2014) who demonstrated that inhibition of NH_4^+ oxidation had no impact on estrogens' biodegradation and deduced that enzymatic reactions which follow NH_4^+ oxidation (i.e., NO_2^- oxidation) do not contribute to estrogen biodegradation. At the same time, Gaulke et al. (2008) affirmed that pure cultures of AOB (*Nitrosomonas europaea* and *Nitrosospira multiformis*), were not able to degrade EE2 under NH_4^+ concentrations of up to 10 mg NH_4^+-N/L. At higher concentrations of NH_4^+ (200-500 mg NH_4^+-N/L), EE2 (at 500 ng/L) was transformed by *N. europaea* but its removal was related to abiotic nitritation owing to the production of NO_2-N and not to enzymatic attack by AMO. The study concluded that the apparent degradation of EE2 reported in previous studies (at initial NH_4^+ concentrations of 50-1400 mg NH_4^+-N/L) was, in fact, abiotic nitritation and heterotrophic biomass was the main responsible for EE2 removal in a full-scale plant. Here, abiotic nitritation was not confirmed since no significant difference in the removal of EE2 (or any other estrogen) was observed between the NAS (no presence of NO_2^-) and the AOB biomass (NO_2^- was the final product of NH_4^+ oxidation), despite the presence of NH_4^+ at concentrations $\geq$ 50 mg NH_4^+-N/L in some of the experiments.

6.3.2 The role of heterotrophic bacteria in EDCs' elimination

6.3.2.1 CAS batch degradation experiments

The experiments detailed in this section aimed to determine the capability of CAS, where heterotrophic bacteria are the predominant species, towards EDCs' removal. The removals achieved, under the studied experimental conditions, are presented in Figure 6.3.

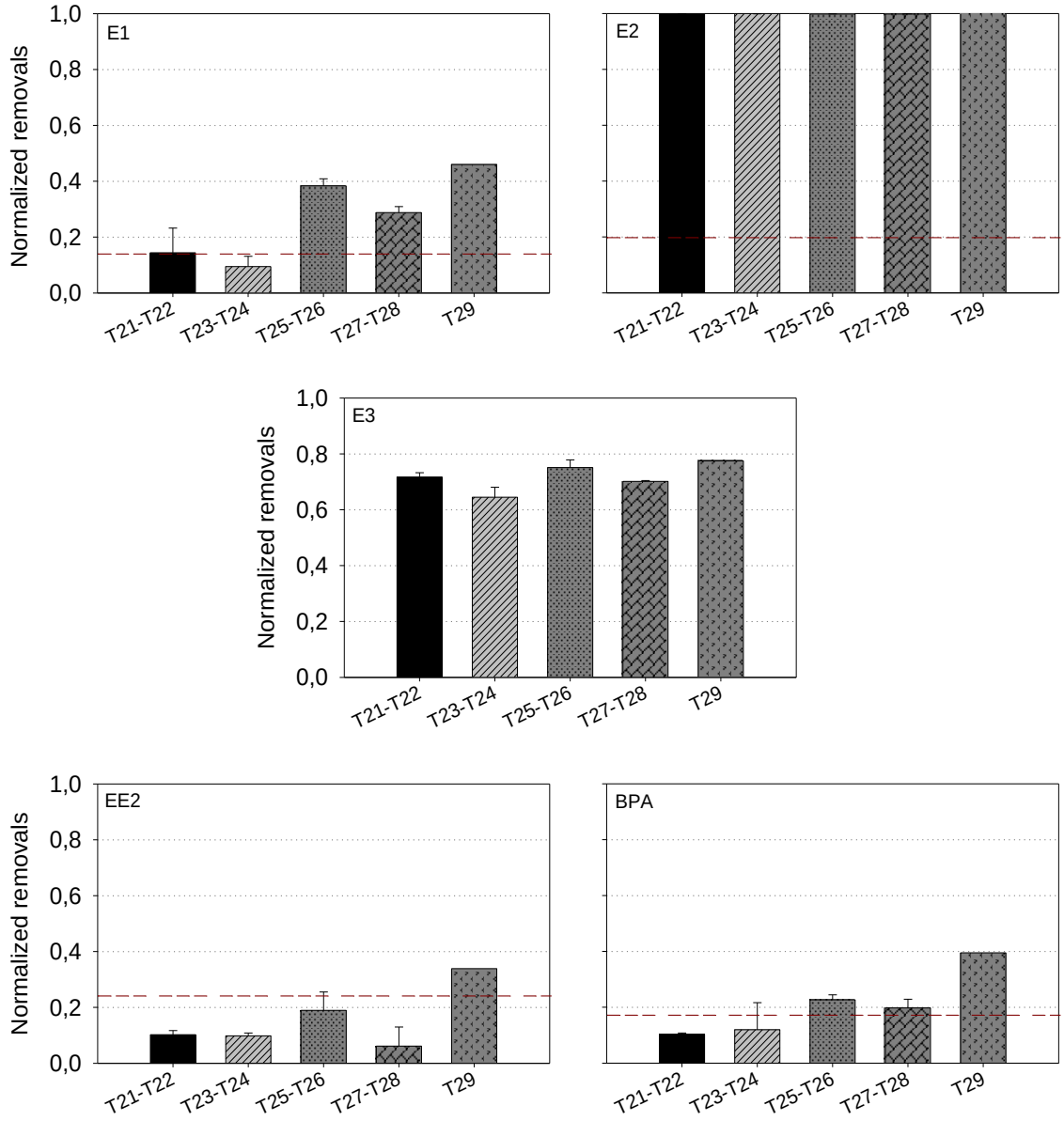


Figure 6.3: Average of normalized removals (C_o-C_{end}/C_o) of E1, E2, E3, EE2 and BPA under the presence or absence of different substrates and ATU (T21-T22: only EDCs; T23-T24: NH_4^+; T25-T26: acetate; T27-T28: NH_4^++ACE; T29: NH_4^++acetate+ATU). The error bars represent standard deviation. Dashed horizontal lines represent the average removal that could be attributed to sorption (more details are provided in 6.3.2.3).

CAS removed E2 and E3 at a high extent (up to 100% and 78%, respectively) whereas EE2 and BPA were found to be more resistant (removals ranged from 6 to 34% and from 10% to 39%, respectively). This behavior was anticipated based on the different degradation rate constants that were identified in literature as explained in the previous section. In fact, each compound displayed a specific range of removals likely related to its degradation capability. E2 and E3 displayed very high removals in all the experiments, with no or slightly noticeable differences. EE2 and BPA, conversely, exhibited low removals and with no remarkable enhancement or deterioration under the diverse conditions applied. High elimination can also be considered for E1 given the fact that it was found to be the main transformation product of E2 (almost quantitative oxidation) as discussed in Section 6.3.2.2.

The recalcitrant behavior of EE2 was expected since it is engineered from E2 by adding an ethinyl group at C-17 position resulting in a compound that is much more resistant to biodegradation (Clouzot et al., 2008). Indeed, its persistence has been confirmed by a number of studies presented by different authors (Hamid and Eskicioglu, 2012; Petrie et al., 2014; Shi et al., 2004; Ting and Praveena, 2017). As regards to BPA, its refractory nature has previously been reported (Ying et al., 2003) while it was also found that the contribution of heterotrophs to its removal is limited (Kim et al., 2007). Moreover, the removal observed for these two compounds could -in some cases- be attributed solely to sorption as discussed in Section 6.3.2.3. In contrast, E2 was always 100% removed independently of the conditions applied. As previously explained, its high biodegradability in comparison to other estrogens, seems to drive its elimination. In fact, fresh CAS biomass readily oxidized E2 under aerobic conditions at both concentrations of 1 mg/L and 1 μg/L, and more than 95% removal was displayed after 1-3 h (Ternes et al., 1999a) whereas EE2 at the same initial concentrations was degraded to only 20% within 48 h and 24 h, respectively.

Different removals were observed for E1 with different substrate addition. In the first two cases, in the complete absence of substrates (experiments T21-T22) or in the presence of NH_4^+ as a sole substrate (experiments T23-T24), the lowest removals (≤ 14%) were detected. Interestingly, in the presence of acetate either in combination with NH_4^+ (experiments T27-T28) or as a sole substrate (experiments T25-T26), higher removals of 29 and 38% with acetate consumptions of 14.0 and 18.2 mg ACE/L/h were exhibited, respectively. These results highlight the biodegradation capabilities of the heterotrophic fraction of the biomass, conforming to our previous findings on the poor performance of NAS and AOB. This

hypothesis is further reinforced by the experiment in the presence of the inhibitor ATU (experiment T29). Although nitrification was inhibited (no NH_4^+ oxidation and no formation of NO_3^- were detected), acetate consumption was not affected (18.7 mg ACE/L/h) and the removals obtained were equally high for E2 and E3 (and similar or higher for E1, EE2 and BPA) in comparison to those without ATU, confirming the contribution of the heterotrophic fraction. These findings accord well with those of Petrie et al. (2014) where, although ATU inhibited the nitrifying activity, the heterotrophic activity remained unafected and comparable biodegradation rates were achieved for the studied estrogens by CAS.

In the present study, the obtained removals with CAS under environmentally relevant concentrations (ng/L to µg/L as stated by Forrez et al. (2009)) of EDCs are promising.

6.3.2.2 E2 and E1 correlation

Both Ternes et al. (1999) and Lee and Liu (2002) affirmed that E1 was the major metabolite of E2 in batch experiments performed with CAS and reported a quantitative oxidation with little and/or no other major degradation products observed.

Independent experiments were thus carried out in order to confirm the relationship between E2 oxidation and E1 formation by CAS under the selected experimental set-up and conditions. For this purpose, only E2 was spiked in the system. Results revealed that E1 was not quantitatively produced since in both experiments, a complete removal of E2 led to a normalized E1 production of 0.1 (Figure 6.4a). This suggests either a further -almost complete- oxidation of E1 (up to 90%) or other possible unidentified E2 TPs.

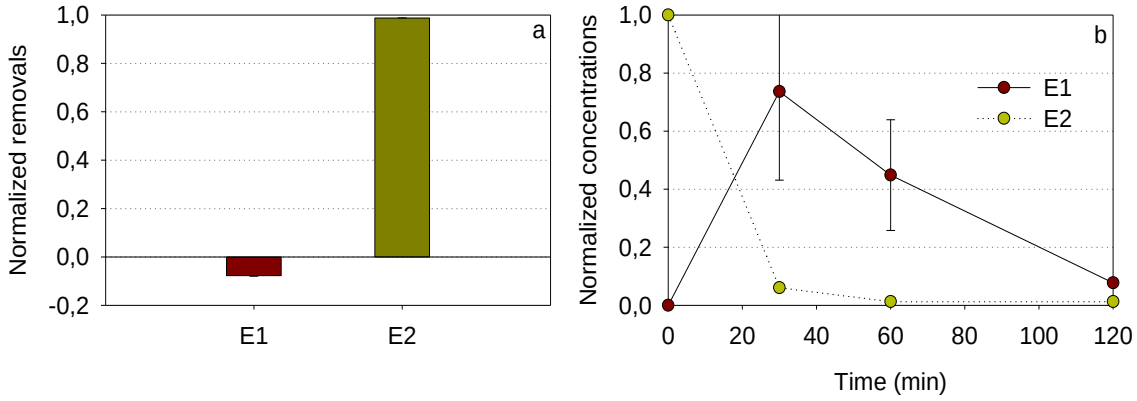


Figure 6.4: E2 and E1 independent experiments with CAS; a) average of normalized removals (C_o-C_{end}/C_o) and b) time course of normalized concentrations (C_x/C_o) of both compounds over time.

The former rather than the latter hypothesis seems to be correct since the degradation kinetics depicted in Figure 6.4b demonstrate that the increase in E1 concentration developed opposite to that of E2. After 30 min an almost quantitative formation of E1 was displayed followed by an almost complete depletion by the end of the experiment. These findings demonstrate that E1 was found to be the main transformation product of E2 in accordance with previous studies, and underline the estrogen degrading capacity of CAS. However, further possible TPs neither of E1 nor of E2 were identified as this was beyond the scope of the present study.

6.3.2.3 Sorption CAS experiments

Different batch and full-scale studies indicated biodegradation and sorption of estrogenic compounds in activated sludge systems as possible removal pathways (Bagnall et al., 2012; Hamid and Eskicioglu, 2012; Li et al., 2011; McAdam et al., 2010), with biodegradation being, however, more significant. Indeed, many researchers reported that sorption typically accounts for less than 10% of estrogens' removal from wastewater, with only two studies mentioning removal of up to 30% in the case of EE2 (Racz and Goel, 2010). E2, EE2 and BPA sorption to activated and to inactivated CAS sludge from WWTPs was also studied, indicating that within a contact time of 2 or 24 h, no significant difference between the sorption to these two types of sludge could be detected (Clara et al., 2004; Zeng et al., 2009). Therefore, sorption experiments were carried out in the present study with heat-inactivated biomass in order to elucidate which part of the removal displayed in CAS experiments could be attributed to sorption.

The results unveiled that sorption was not the main mechanism of elimination as it accounted for 0 to ≤ 20% in the case of E1, E2 and E3. However, it was found to be a significant route for EE2 and BPA because sorption removals were similar to the total ones observed during the CAS experiments. More specifically, from the total removal achieved in each experiment that was presented in Section 6.3.2.1 the following amounts (expressed as normalized removals) could be attributed to sorption (Table 6.2).

Table 6.2: Sorption removals for each one of the studied compounds

	E1	E2	E3	EE2	BPA
Average removal	0.14	0.20	-0.05	0.24	0.17
SD	0.13	0.09	0.06	0.04	0.02

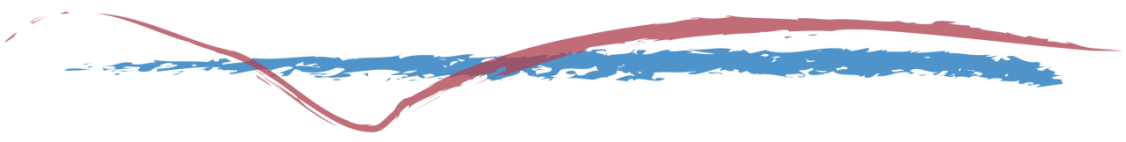
Wang et al. (2013) reported that EE2 had the strongest sorption affinity followed by E2, E1 and finally E3. Similar results were obtained in experiments with aquifer material and the studied compounds followed the order EE2 > E2 > BPA (Ying et al., 2003). The results here acquired are in line with the literature and support the notion that sorption could be a significant removal mechanism, even though limited, for recalcitrant compounds such as EE2 and BPA, as also supported by Cajthaml et al. (2009).

Overall, the results obtained during the experiments with CAS are in accordance with literature. Baronti et al. (2000), Ternes et al. (1999b) and Andersen et al. (2003) investigated the fate of estrogenic compounds in many municipal WWTPs with activated sludge systems and reported average removal efficiencies of 61-98%, 87-99.9%, 95% and 78-90% for E1, E2, E3 and EE2, respectively. It is thus confirmed that CAS, which is the most widely used type of biomass in secondary treatment for municipal and industrial wastewater treatment, shows better attenuation of estrogenic hormones compared to other biological treatment options as also stated by Hamid and Eskicioglu (2012).

Block III

General Discussion, Future Perspectives and Conclusions

General Discussion



7.1 The problematic behind micropollutants

Surface and groundwater are considered as valuable resources for drinking water supply. At the same time, rivers, lakes, wetlands and oceans along with their associated ecosystems provide natural habitat to plant and animal species, upon which future generations rely for their survival. Nowadays, water stress issues undermine prosperity. Beyond water scarcity, micropollutants have emerged as a new threat and consequently a challenge, for the scientific community.

The occurrence and continuous input of micropollutants, such as PhACs and EDCs, into the environment owing to their massive production are neither particularly monitored nor controlled, which raises concerns to Water Utilities as many drawbacks have to be overcome. The significant spatial and temporal variations that PhACs and EDCs reveal in combination with their diversity and continuous transformation in the human body and during sewage transport and treatment, complicate their associated detection (especially at trace concentrations), and the assessment of their toxicological significance. Exposure to complex mixtures could be more alarming owing to synergistic effects, while the potential stability or toxicity of such compounds is another issue to be considered. As a result, regulation of these substances and their possible TPs is challenging and a legal framework for drinking water and wastewater has not yet been established despite the fact that they are recognized as potential hazards for ecosystems and humans (Boxall, 2004; Houtman, 2010).

7.2 Fate in different treatment systems

The fate of micropollutants during biological wastewater treatment is determined by their physicochemical properties, by biomass capabilities and by wastewater treatment-related factors (i.e. HRT, SRT, redox conditions, pH and temperature). The large variations among each of these factors lead to obvious repercussions on the behavior and removal efficiencies of these compounds.

The present thesis investigated the fate of five PhACs (IBU, SFX, MTP, CBZ and VEN) and five EDCs (E1, E2, E3, EE2 and BPA) of concern in different engineered treatment systems performing: nitrification, partial nitrification (i.e. nitritation) and combined partial nitritation-anammox, while the contribution of the microbial groups thriving in such systems was also

determined. The results obtained in the three chapters of the present thesis confirmed the hypothesis that many factors affect and control the removal of these compounds.

7.2.1 Factors governing the removal of micropollutants

Compound biodegradability

Compound structure complexity and functional groups are factors to be considered in determining PhACs and EDCs susceptibility towards biodegradation. The first phase of the biodegradation process is, in fact, the uptake of micropollutants by the cell, regulated by chance affinity of the compound with the bacterial enzymes (Siegrist et al., 2005).

For example, E2 and E3 (in the case of CAS experiments, Chapter III) as well as IBU (Chapter II) exhibited high removals under all or most, respectively, of the diverse conditions applied, demonstrating their high biodegradability. E2 and IBU were removed up to 100%, whereas E3 up to 78%. Conversely, the recalcitrant character and persistence of CBZ, VEN, EE2 and BPA (Chapter II and III) was displayed and no remarkable removals were observed in any of the experiments, as previously confirmed by other studies (Falås et al., 2013; Hamid and Eskicioglu, 2012; Ying et al., 2003). In line with these findings, highly or poorly biodegradable compounds showed negligible dependence on environmental conditions with their degradation being always high or trivial in the first and latter case, respectively (Sgroi et al., 2017). Nevertheless, in some cases, there is no obvious relationship among chemical structure, functional groups and removal, and two structurally similar compounds such as IBU and ketoprofen could show different degrees of elimination (Luo et al., 2014).

Unique biomass capabilities

Each biomass has unique metabolic capabilities that, together with the diverse operational conditions, control and affect the transcript and enzymatic pools and could consequently lead to different removal efficiencies (Helbling et al., 2012). For example, although E2 and E3 were greatly removed during batch experiments with CAS, an almost negligible removal was displayed in the experiments with both enriched AOB and NAS. Additionally, contrary to previous findings (de Graaff et al., 2011; Laureni et al., 2016) higher removal efficiencies were obtained for MTP (Chapter II) during the regular combined N/A operation and optimal conditions for anammox bacteria, pointing out that the growth of

diverse microbial populations even in similar treatment systems can lead to different degradation capabilities.

Redox conditions

In Chapter II it was demonstrated that the biodegradability of a compound is not the only driving force, and that redox conditions in combination with the active microbial group involved contribute in a significant way. IBU displayed a null removal under anoxic conditions aiming to heterotrophic denitrification, despite its high biodegradability. Additionally, anoxic conditions were found to be relatively more favorable in the case of SFX, in line with the findings of Sgroi et al.,(2018). However, under optimal conditions for nitrifying bacteria (aerobic) a high removal of 70% was observed for SFX, indicating that in this case the removal was not affected by the redox conditions but rather by the microbial group that was favored, or by the nitrification rate.

Nitrification rate

In Chapter I, nitrification rate was found to be a critical factor in the removal of SFX, with higher degradations (up to 91%) being obtained at higher rates. In Chapter II, a comparison between the experiment simulating nitrification (exp. ii) and that of combined N/A (exp. i) reinforced this idea, as IBU, SFX and MTP were removed at a much higher extent in the first case with a nitrification rate of 5.28 mg NH_4^+-N/min as compared to the latter case where a much lower rate was achieved (0.39 mg NH_4^+-N/min). The removals obtained were 81 and 49%, 70 and 17% as well as 37 and 20% during the experiment ii and i and for IBU, SFX and MTP, respectively. Indeed, higher removal rates of some PhACs and EDCs have been reported in nitrifying systems. A positive correlation has been demonstrated at increasing rates owing to higher AOB activity most probably related to a higher expression of AMO (Alvarino et al., 2014; Dytczak et al., 2008; Fernandez-Fontaina et al., 2012; Ren et al., 2007; Tran et al., 2009; Yi and Harper, 2007). As a matter of fact, amoA mRNA level was found to dynamically change in response to the changes in NH_4^+ concentration during batch-mode incubation and was proposed as an accurate biomarker of the NH_4^+ oxidation activity in engineered systems with complex bacterial consortia (Aoi et al., 2004).

Contrariwise, increasing or decreasing nitrification rates did not enhance or deteriorate the removal of the studied compounds during some of the experiments in Chapters II and III. E1, E2, E3, EE2 and BPA exhibited very low removals (< 14%) and showed no correlation with

the increasing nitrification rates in both enriched NAS and AOB biomass (Chapter III). At the same time, in the case of anaerobic ammonium oxidation (exp. iv), IBU, SFX and MTP exhibited higher removals (55%, 58% and 62% respectively) as compared to those achieved during the combined N/A operation (49%, 17% and 20% respectively), although the nitrification rate was lower (0.25 mg NH_4^+-N/min), pointing out the degradation capabilities of anammox bacteria (Chapter II).

7.2.2 Formation of TPs

Different behavior was also observed in the case of the studied TPs that were evaluated in parallel to the degradation of the parent compounds. As regards to SFX in enriched AOB biomass, TPs formation represented around 30% of the initial mass, being the main one 4-NO_2-SFX and with a minor formation of Desamino-SFX and N^4-Acetyl-SFX (Chapter I). Interestingly, although SFX was highly removed, the formed TPs could not be further degraded nor were cleaved back to SFX as demonstrated by independent experiments. This result raises concerns as SFX TPs can present similar of higher ecotoxicological effects compared to SFX (Majewsky et al., 2014). On the contrary, 4-NO_2, Desamino and N^4-Acetyl-SFX were always below LOD or LOQ (Table 3.2) in the combined N/A microbial community and under all the conditions applied (Chapter II). Similarly, the studied TPs of MTP, CBZ and VEN were also below LOD or LOQ and only 1-hydroxyl-IBU and 2-hydroxyl-IBU were averagely formed at 0.66 ± 0.59 μg/L and 0.8 ± 0.54 μg/L respectively. The results stress that the fate of TPs is also difficult to assess. Only a comprehensive monitoring of metabolites, TPs as well as end products of mineralization can provide information about the real degree of biotransformation of the parent compounds, which is of high importance given the possible stability and toxicity of these by-products as well as of complex mixtures (Kümmerer, 2009; Ternes et al., 2007).

7.3 How close are we to solving the puzzle?

The present thesis investigated the fate of PhACs and EDCs in different systems simulating full-scale treatment scenarios and identified factors triggering their elimination as well as the potential of diverse microbial groups. Although promising removals were obtained in some cases, none of the compounds could be efficiently eliminated under all the treatments and conditions applied. The fate of such a broad range of compounds cannot be

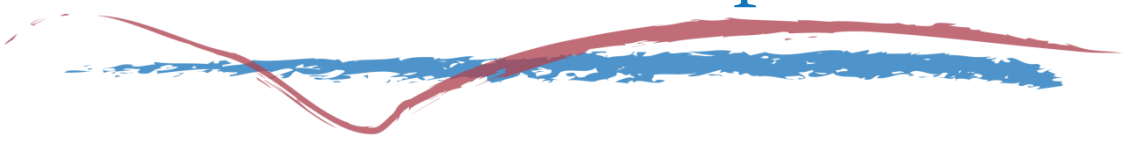
easily predicted as the unique degradation capabilities of some microbial groups and/or the multiple experimental conditions are not always easy to understand or to control. At the same time, while the overall efficiency can be broadened by combining different aerobic and anaerobic treatments and different types of biomass, some micropollutants will still persist rendering biological approaches ineffective and results sometimes inconsistent. Nonetheless, they could still be used as a first barrier which, coupled (if applicable) to an advanced treatment, could meet the expected stringent discharge limits in terms of micropollutants removal. TPs formation should also urgently be considered as concerns arise regarding their toxicity and potential stability.

In accordance to these findings and moving to full-scale systems, similar observations have been made. Up-to-date results remain inconsistent and no method that could assure efficient elimination, by means of biodegradation, of the majority of PhACs, EDCs and their TPs has been found. In a point of fact, the removal efficiency of different WWTPs was found to vary greatly and ranges of 72-100%, 4-88.9%, 3-56.4%, 0-62.3%, ≈25-50%, 74.8-90.6%, 90-100%, 99-100%, 43.8-100% and 62.5-99.6% for IBU, SFX, MTP, CBZ, VEN, E1, E2, E3, EE2 and BPA (Table 1.2) have been detected. However, neither the number of the presented WWTPs nor that of the selected compounds (in Table 1.2) can be considered a representative sample taking into account the great variability within installations as well as the thousands of existing PhACs and EDCs that would of course exacerbate the problem. At the same time, although novel treatments (such as advanced oxidation, adsorption and membrane processes) generally show more efficient and consistent removal of micropollutants, retrofitting most of the WWTPs with such technologies will not be acceptable as consistent investment at reasonable operational costs, whereas formation of by-products and concentrated residues is another critical issue to be considered (Luo et al., 2014; Schröder et al., 2016).

Thus, prior to a broad upgrading of WWTPs it would be prudent to quantify the risks associated with the current effluent quality. Emphasis should be given on ecotoxicological studies dealing with the environmental risk assessment of mixtures of relevant (based on presence, quantity and persistence) substances to not target organisms. One approach might be that WWTPs discharging into receiving waters either used for human activities (e.g. for drinking water production or bathing activities) or with sensitive aquatic organisms have to fulfill higher quality standards (Ternes and Joss, 2015).

It can thus be deduced that although a lot of essential knowledge has been gathered within the last decades there is still much room for research since many gaps need to be filled.

Future Perspectives



Up-to-date, conventional WWTPs cannot cope with the elimination of micropollutants, despite several advances since the inception of biological wastewater treatment processes (Singhal and Perez-Garcia, 2016). The degree of removal greatly depends on the type of treatment, applied conditions, microbial communities present and variety and concentration of xenobiotic substances in the system, as demonstrated by a great number of studies including the present thesis. At the same time, environmental risk assessment studies for several PhACs and EDCs along with their metabolites are yet to be performed as the cocktail effect is difficult to evaluate and we should also focus our efforts and attention in this field.

In the meanwhile, finding alternative solutions is critical. Looking from a temporal perspective many types of action could be taken and seem to either focus on the addition of a quaternary advanced treatment or on an ultimate effort of optimization of the existing technologies. In the first case, two main technologies with a potential for large-scale application in terms of efficiency, cost and energy requirements can be identified: i) oxidation of micropollutants with ozone and ii) adsorption onto activated carbon (Margot et al., 2015). Indeed, in the case of Switzerland where the government made a legally binding decision on upgrading WWTPs in order to reduce the loads of micropollutants, these two methods have been selected as a reliable approach that could assure substantial elimination of $\geq$ 80% for specific compounds (Logar et al., 2014). In the second case, the ultimate evolution of biological wastewater treatment processes could be based on: i) discovering the possibilities of known enzymes (e.g. AMO) that have a broad substrate range and have been found to be capable of oxidizing a large variety of pollutants (Tran et al., 2013) and ii) discovering and employing novel metabolic traits of unconventional microbes, or inducing the synthesis of novel enzymes capable of degrading micropollutants (Singhal and Perez-Garcia, 2016). Indeed, the degradation capabilities of different classes of enzymes, such as i) the oxygenase Cytochromes P450, a highly efficient group of monooxygenases responsible for the destruction of drugs and toxins in organisms and ii) the laccases, a class of copper-containing oxidizing enzymes used by microorganisms to break down lignin (Kumar, 2010; Riva, 2006), has been demonstrated in pure enzyme cultures (Harms et al., 2011; Lah et al., 2011).

On the other hand, from a long-term perspective, a change of mindset might be necessary to tackle this problem.

Personal view

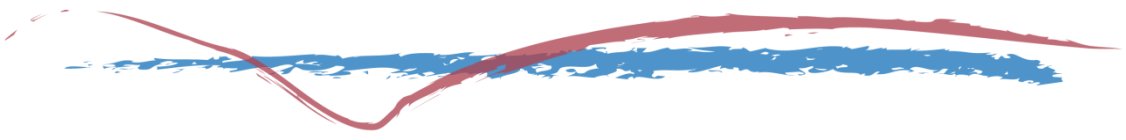
A doctoral thesis is about filling in some research gaps along the way. Personally, I was hoping that by the end of this journey I would have tackled some of the main questions and doubts related to the removal of micropollutants and that this thesis would be an important contribution -although a drop- to the ocean of research studies and knowledge. Although I have put a lot of effort and love to produce the document you are reading today, I can't deny that it feels like a piece of the puzzle is missing.

The mission of scientists and engineers working on the occurrence and fate of micropollutants in the field of urban water and waste (or resource –as the new name will eventually be) water, is to provide and implement innovative solutions which could integrate across different aspects to assure environmental sustainability and public health protection. Towards this direction, the knowledge gathered in the present thesis is relevant and in combination with the aforementioned promising approaches will contribute in the implementation of environmental policies to create a barrier to micropollutants emissions.

However, the solution should not be a one-way road. Indeed, alternative control strategies different to end-of-pipe solutions, such as segregation of sources, improvement of drug disposal, more considerate prescription of PhACs and EDCs by doctors to limit unnecessary overuse or abuse, users awareness and promotion of best management practices are already under investigation. Notwithstanding, the thousands of pharmaceutical compounds being in use along with their unknown transformation products, the great uncertainty regarding their fate and mainly the potential and still undefined risks that such complex mixtures can pose, indicate that perhaps we are not that close to solving the problem yet. Perhaps one of the best solutions would hence be the use of environmental friendly and biodegradable substances (i.e. promotion of green chemistry and ecolabeling) but much effort is still to be done mainly due to lack of willingness and political headwinds.

The road of success thus seems to be long and winding but we can all engage constructively in seeking the best strategies to lead us through it. Definitely, a multidisciplinary approach is the best way to address such a complex matter.

General Conclusions



The present thesis investigated the fate of five PhACs (IBU, SFX, MTP, CBZ and VEN) and five EDCs (E1, E2, E3, EE2 and BPA) of concern in different lab, pilot and full-scale treatment systems performing: nitrification, partial nitrification (i.e. nitritation), combined partial nitritation-anammox and aerobic oxidation of COD. To determine the contribution of the autotrophic and heterotrophic bacteria present (in such systems) in the removal of the selected compounds, several experiments were carried out by selectively activating (presence of substrates and optimal conditions) or inhibiting (absence of substrates and/or presence of inhibitors) the different microbial groups. A comparative assessment of their capabilities was carried out, whereas factors triggering the elimination of the studied compounds were also identified.

A summary of the main conclusion of each Chapter of the thesis is presented below:

Chapter I

Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products

Experiments were carried out with enriched AOB biomass cultivated at lab-scale in order to investigate its degradation capacity in removing SFX. The main outcomes of the present study can be summarized as follows:

- Enriched AOB biomass demonstrated high degradation capabilities in the elimination of SFX even without a previous period of adaptation.
- SFX was degraded up to 86% and up to 98%, during aerobic short term (contact time of 6 hours, batch mode) and long term (contact time of 24 hours in the SBR-PN) experiments, respectively.
- SFX degradation was clearly related to the nitrification rate with higher removals being obtained under higher SAORs. Three zones of different SAORs corresponding to different removals were identified. Nevertheless, also with nitrification decreased or suppressed due to the lack of NH_4^+, SFX degradation was still observed at around 25%.
- In the presence of ATU nitrification was completely inhibited and SFX was not degraded.

- An efficient UPLC-QqLIT method for the determination of 4-NO_2 SFX, Desamino-SFX and N^4-Acetyl-SFX in batch samples without sample pre-concentration has been optimized and validated with good quality parameters.
- TPs formation represented around 30% of SFX in the short and long term experiments conducted. In both cases, the main TP was 4-NO_2-SFX, whereas the formation of Desamino-SFX and N^4-Acetyl-SFX was minor. TPs could not be further degraded during independent batch experiments.

Chapter II

Unraveling the potential of a combined nitritation-anammox biomass towards the biodegradation of pharmaceutically active compounds

Experiments were carried out with combined partial-nitritation biomass in order to investigate its degradation capacity and key factors affecting the elimination of IBU, SFX, MTP, CBZ and VEN. Based on the results the following main conclusions are drawn:

- Redox conditions affected mainly the removal of IBU which was greatly removed under aerobic conditions (up to 100%), whereas the removal of SFX was slightly enhanced under anoxic conditions.
- PhACs removal was affected by the nitrification rate. For MTP, IBU and SFX removals dropped by 17%, 32% and 53% respectively in the combined N/A experiment as compared to the aerobic-AOB experiment where a much higher NH_4^+ oxidation rate was obtained.
- In the presence of ATU, IBU and MTP degradation was not or only slightly affected indicating that it was most probably carried out by ATU-insensitive enzymes. In contrast, SFX removal dropped by 53% suggesting the contribution of AOB.
- CBZ and VEN displayed a quite recalcitrant behavior with removals being lower than 10% under most of the conditions tested.

Chapter III

Comparative assessment of endocrine disrupting compounds removal in heterotrophic and enriched nitrifying biomass

Batch experiments were carried out with enriched NAS and AOB biomass, cultivated at lab-scale, as well as with CAS biomass, withdrawn from a municipal WWTP, in order to

investigate their capacity in removing E1, E2, E3, EE2 and BPA. The main outcomes of the present study can be summarized as follows:

- Both enriched NAS and AOB biomass demonstrated a poor performance. The studied estrogens exhibited low removals (< 14%) and showed no correlation with the increasing SAORs.
- The heterotrophic fraction of CAS demonstrated a better attenuation in comparison to NAS and AOB but different degradation capabilities were displayed among compounds.
- E2 and E3 were removed up to 100% and 78%, respectively. Although removals ranged from 10% to 46%, high elimination could also be stated for E1, being the main transformation product of E2 with almost quantitative oxidation as demonstrated by independent experiments. EE2 and BPA were more persistent biologically with removals ranging from 10% to 39%.
- Experiments with heat-inactivated CAS biomass revealed similar removals -to those obtained without inactivation- only for EE2 and BPA, suggesting that sorption could be a relevant route of elimination for these two compounds.

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Annex

Block I:

Section 1.4: Compounds selected for the present thesis: properties, presence and removal

Table S1: Molecular structure and weight of the selected PhACs

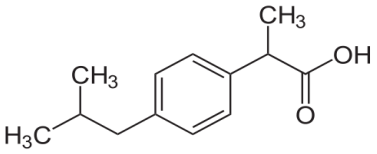
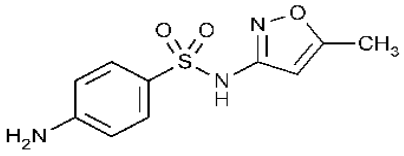
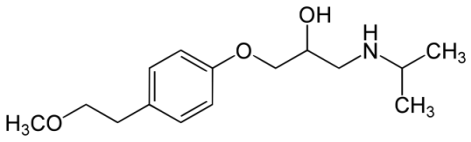
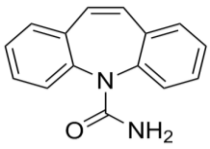
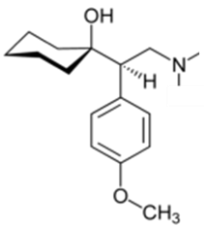
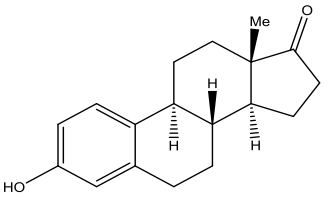
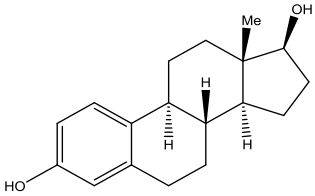
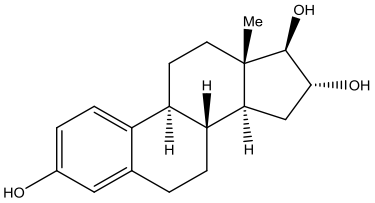
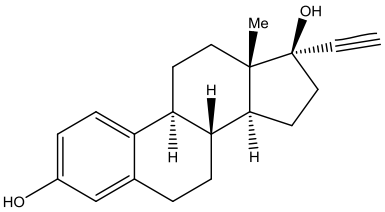
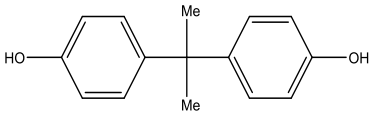
Compound	Molecular Structure	Molecular Weight (g/mol)
Ibuprofen (IBU)		206.3
Sulfamethoxazole (SFX)		253.3
Metoprolol (MTP)		267.4
Carbamazepine (CBZ)		236.3
Venlafaxine (VEN)		277.4

Table S2: Molecular structure and weight of the selected EDCs

Compound	Molecular Structure	Molecular Weight (g/mol)
Estrone (E1)		270.4
17β-estradiol (E2)		272.4
Estriol (E3)		288.4
17α-Ethinylestradiol (EE2)		296.4
Bisphenol A (BPA)		228.3

Block II:

Chapter I

Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products

Section 4.2.1: Bioreactor set-up and operation

FISH analysis showed that AOB bound with the FISH probe Cy3-labeled AOBMIX were dominant (more than 80% of the microbial population corresponded to AOB), as presented in the FISH micrographs (Figure S1).

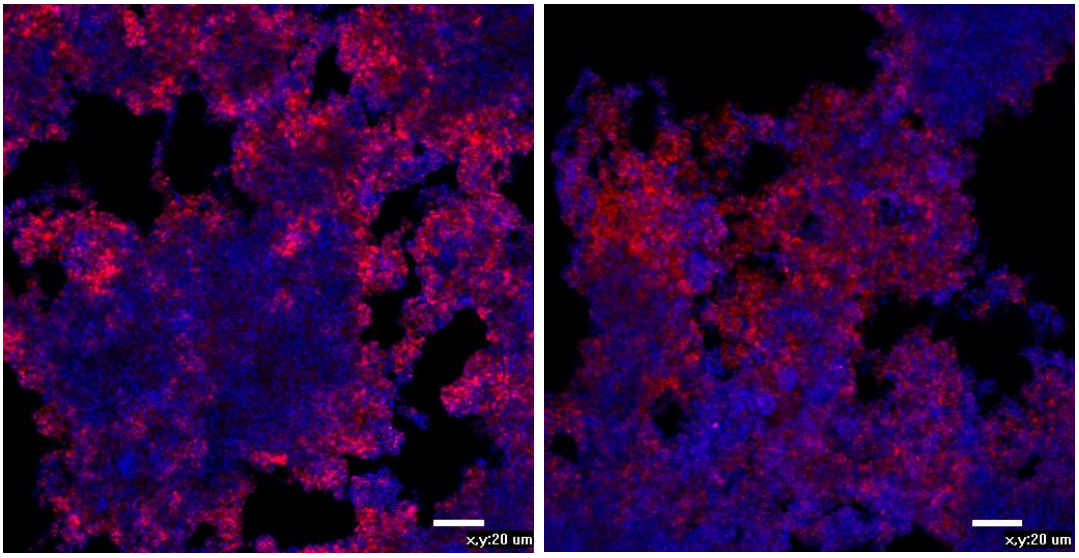


Figure S1: Two representative CLSM micrographs of sludge hybridized with Cy3-labeled AOBMIX probes and Cy5-labeled EUBMIX probes. AOB are shown in magenta color and all other bacteria in blue color.

Section 4.2.2: Batch experiments

Sorption experiment

A sorption control was executed with SFX and activated sludge following the standard procedure. 50 mL of mixed liquor were withdrawn from the parent SBR-PN with a concentration of 2 g/L. The biomass was subsequently washed with a phosphate buffer solution in order to ensure that all the nutrients were removed completely, and was then diluted (1:2) with distilled water to a final concentration of 1 g/L. To ensure total inactivation of the microbial activity, the biomass was autoclaved at 121^{o}C for 20 minutes and dispensed into a 250 mL Erlenmeyer flask. SFX was added to achieve an initial concentration of

100μg/L, and the mixture was agitated by a magnetic stirrer. Aqueous samples were taken along the experiment (at the beginning, after half an hour, after two hours and after four days) and were filtered through 0.45μm PVDF filters. SFX was analyzed as described in Section 0. The results are presented in Figure S2.

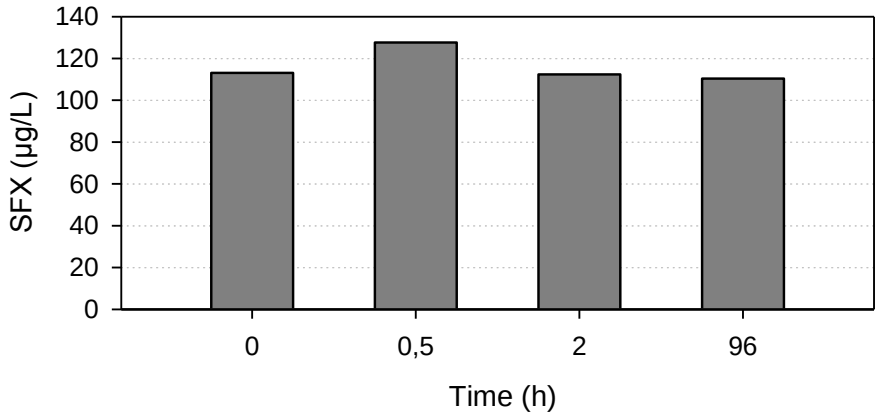


Figure S2: Monitoring of SFX concentration during the four days of the sorption control experiment.

Abiotic experiments

Preliminary abiotic experiments were executed with SFX at different concentrations of NO_2^-. The aim of these experiments was to investigate if any chemical transformation of SFX or chemical formation of 4-NO_2-SFX could occur under different ways of sample preservation (at -20°C and at 4°C, overnight), so as to select the one that could reassure the reliability of our results (Figure S3).

Three experiments were performed with Milli-Q® water containing 200 μg/L SFX and three different concentrations of NO_2^- (200, 50 and 5 mg NO_2^--N /L), while pH was controlled at 7.8. This concentration of SFX was selected accordingly to the initial concentration at time 0, in all the biotic experiments. Additionally, a control experiment with Milli-Q® water containing only 200 μg/L SFX, was executed. All the experiments were conducted in triplicates to obtain accurate results. In each experiment, after the addition of SFX and of NO_2^-, a common sample was taken and split in two parts. One was kept frozen at -20^{o}C, while the other one was stored at 4^{o}C overnight, and were then both injected in the Ultra Performance Liquid Chromatography tandem Mass Spectometry (UPLC-MS/MS) system.

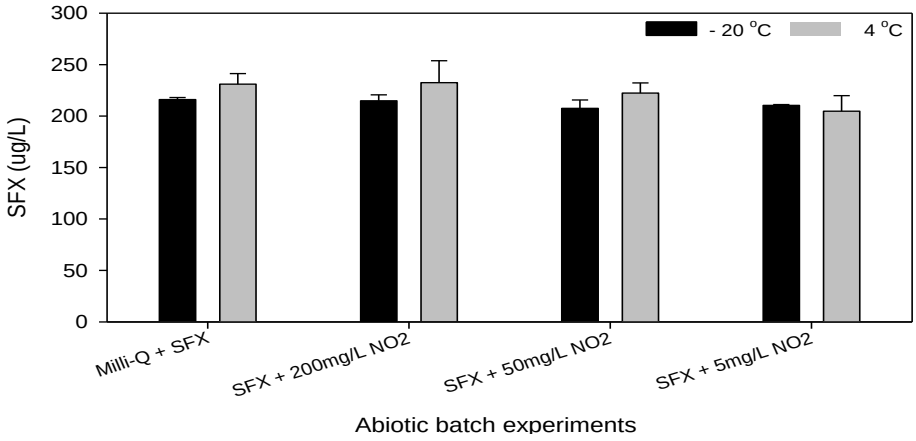


Figure S3: SFX concentration during the abiotic experiments under different concentrations of NO_2^--N and different sample preservation conditions (at -20°C and at 4°C, overnight). 4-NO_2-SFX was not formed in any of the cases. The error bars represent the standard deviation.

Section 4.3.1.1: SFX degradation and TPs formation at different SAORs

A time course of SFX, its TPs and the obtained SAORs are presented for all the experiments in Figures S4-S13.

- Category 1: "NH_4+SFX" (experiments T1-T21)
  - Zone iii) 1.6 ≤ SAOR ≤ 2.1 mg NH_4^+-N/g MLVSS min, corresponding to a range of high removals of 79-91% (average 86±5%). Tests T1-T5.

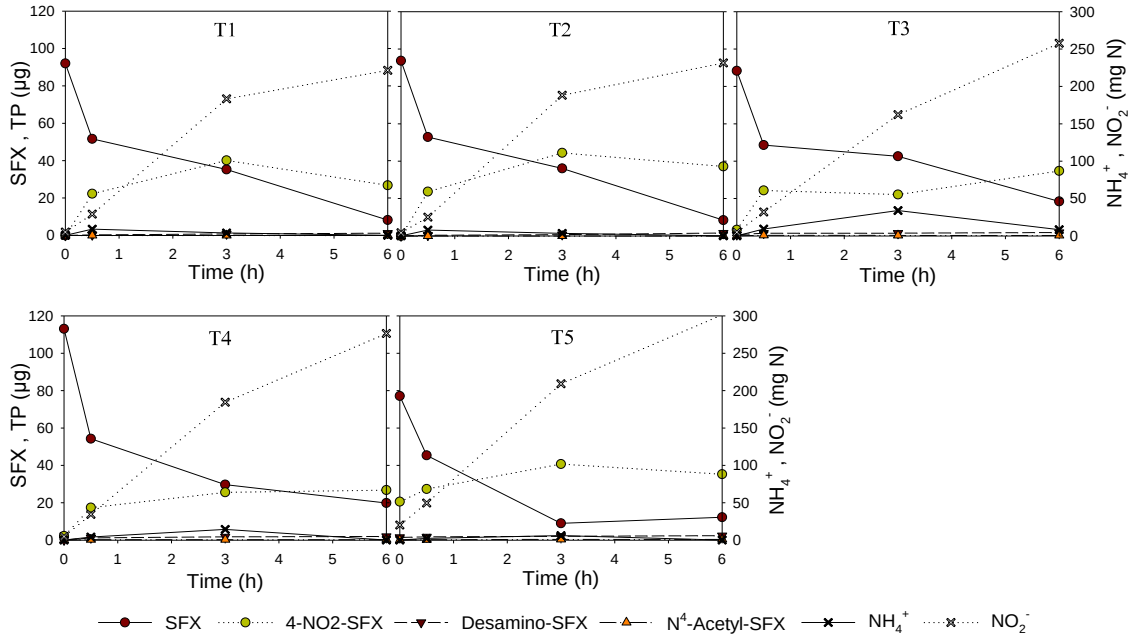


Figure S4: Removal of SFX (µg) and TPs formation during the experiments of category 1 belonging to SAOR zone iii: 1.6 ≤ SAOR ≤ 2.1 mg NH4+-N/g MLVSS min (exp. T1-T5).

- Zone ii) 0.5 ≤ SAOR ≤ 1.2 mg NH4+-N/g MLVSS min, corresponding to a range of removals of 53-83% (average 67±12%). Experiments T6-T15.

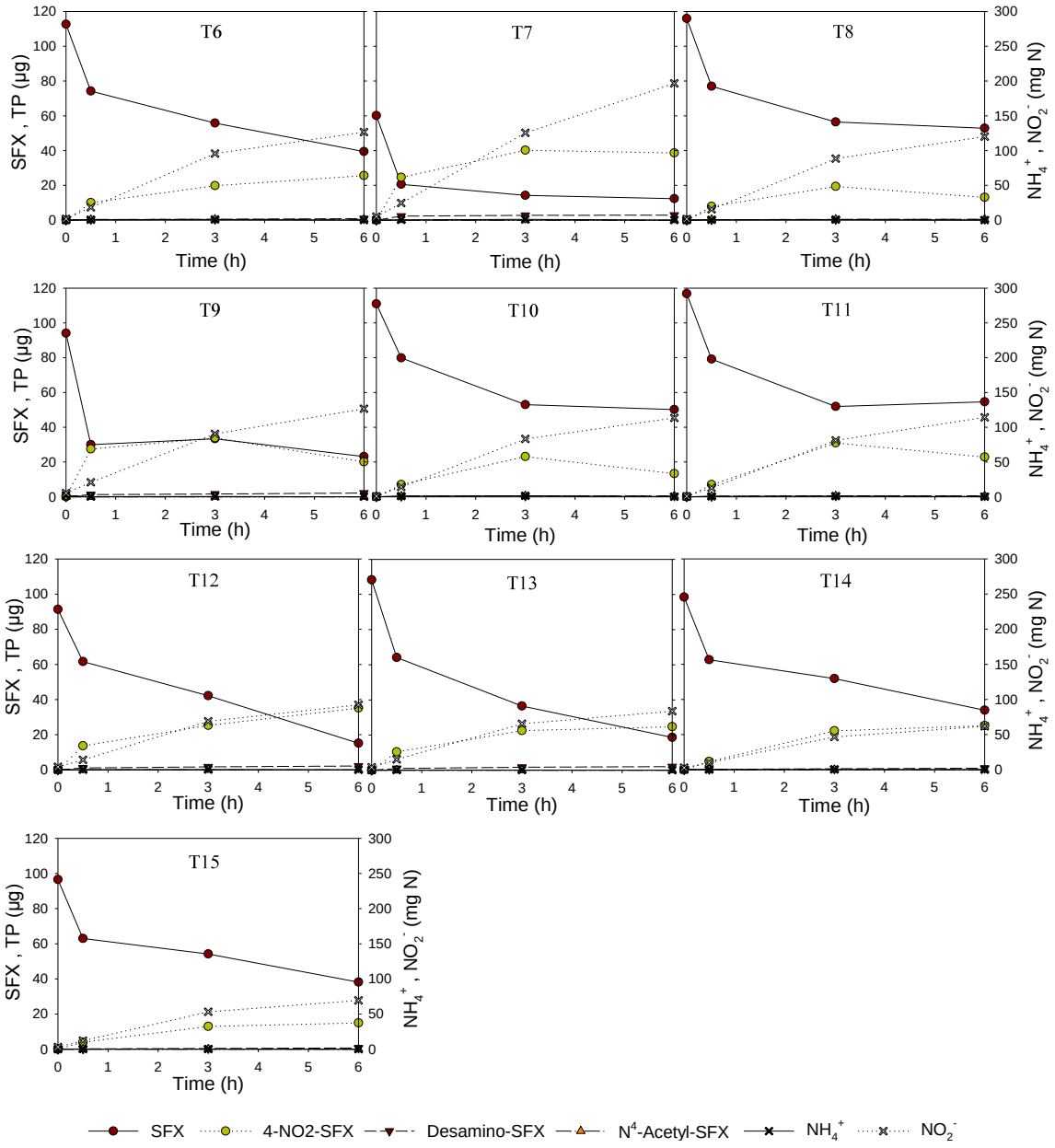


Figure S5: Removal of SFX (µg) and TPs formation during the experiments of category 1 belonging to SAOR zone ii: 0.5 ≤ SAOR ≤ 1.2 mg NH_4^+-N/g MLVSS min (exp. T6-T15).

- Zone i) 0 ≤ SAOR ≤ 0.4 mg NH4+-N/g MLVSS min, corresponding to a range of limited removals of 16-33% (average 25±7%). Experiments T16-T21.

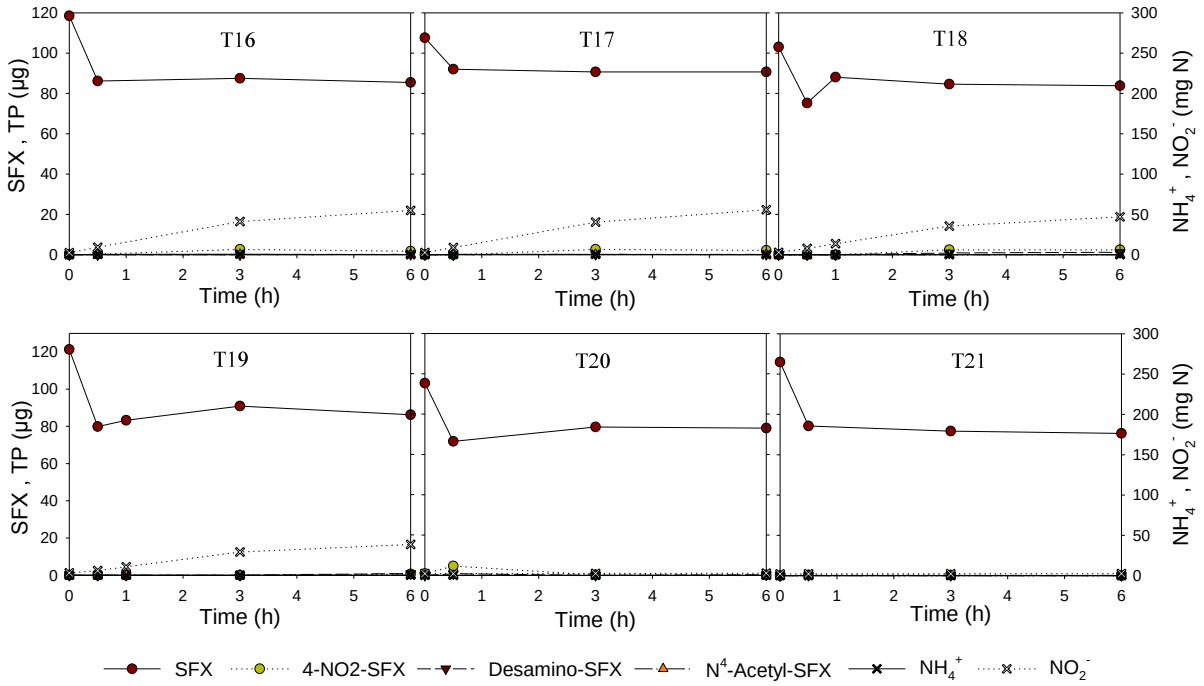


Figure S6: Removal of SFX (µg) and TPs formation during the experiments of category 1 belonging to SAOR zone i: 0 ≤ SAOR ≤ 0.4 mg NH_4^+-N/g MLVSS min (exp. T16-T21).

➢ Category 2: "NH_4+SFX+ATU/ACE" (Experiments T22-T29)

- NH_4^+ + ATU. Experiments T22-T23.

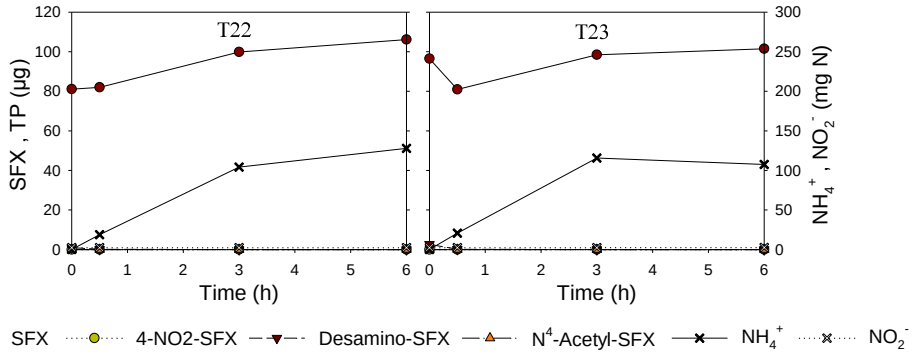


Figure S7: Removal of SFX (µg) and TPs formation during the experiments of category 2 with NH_4^+ and ATU (exp. T22-T23).

- NH_4^+ + ATU + ACE. Experiments T24-T25.

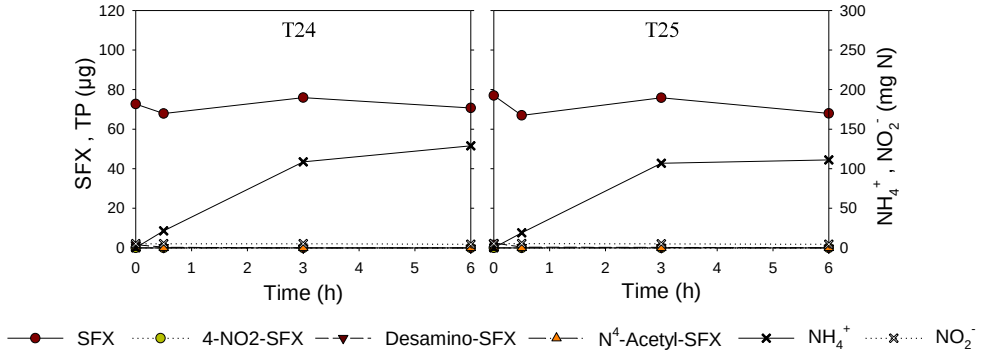


Figure S8: Removal of SFX (µg) and TPs formation during the experiments of category 2 with NH_4^+, ATU and ACE (exp. T24-T25).

- NH_4^+ + ACE. Experiments T26-T27.

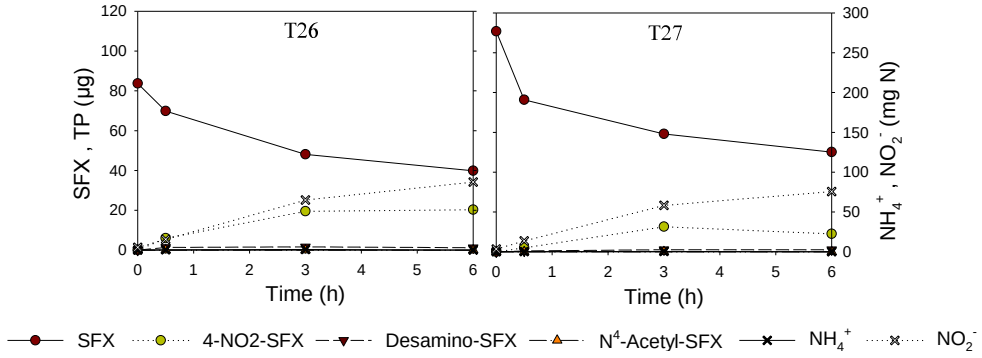


Figure S9: Removal of SFX (µg) and TPs formation during the experiments of category 2 with NH_4^+ and ACE (exp. T26-T27).

- No NH_4^+ +ACE. Experiments T28-T29.

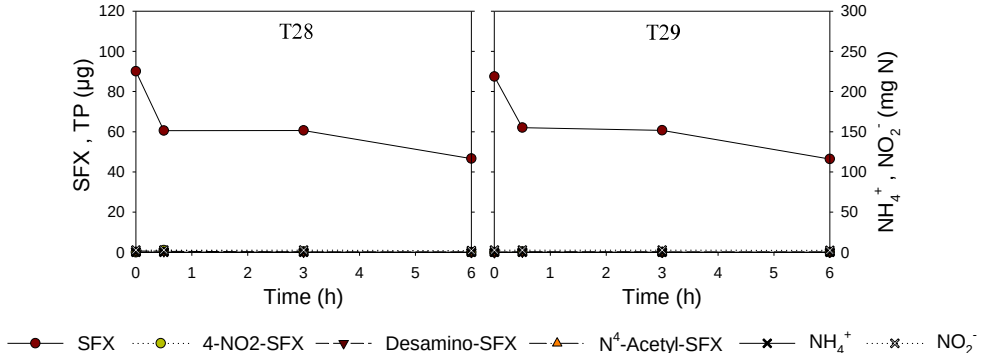


Figure S10: Removal of SFX (µg) and TPs formation during the experiments of category 2 with ACE in the absence of NH_4^+ (exp. T28-T29).

➢ Category 3: "TP" (Experiments T30-T35)

- 4-NO_2-SFX. Experiments T30-T31.

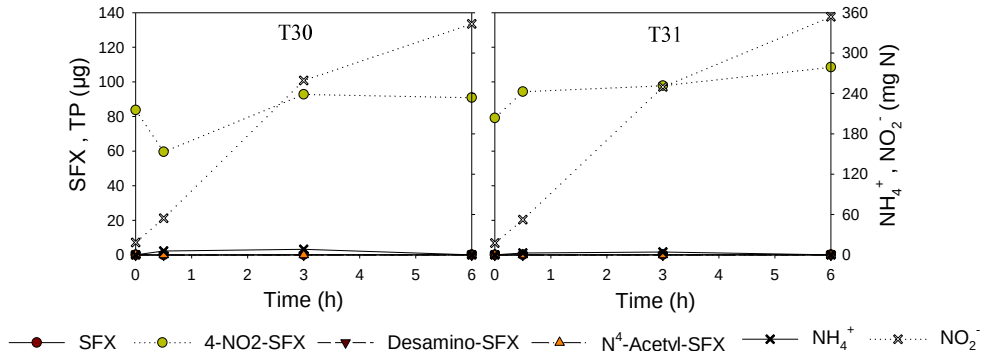


Figure S11: Fate of 4-NO_2-SFX during the independent experiments of category 3 (exp. T30-T31).

- N^4-Acetyl-SFX. Experiments T32-T33.

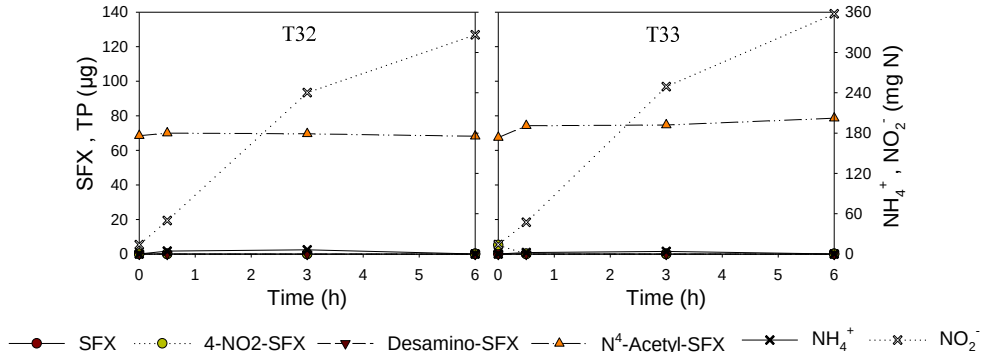


Figure S12: Fate of N^4-Acetyl-SFX during the independent experiments of category 3 (exp. T32-T33).

- Desamino-SFX. Experiments T34-T35.

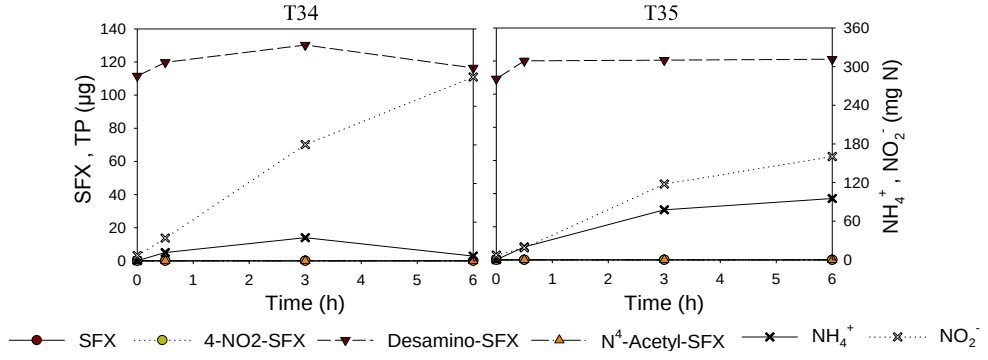


Figure S13: Fate of Desamino-SFX during the independent experiments of category 3 (exp. T34-T35).

Section 4.3.1.2: Formation of TPs

Quality parameters of analytical methods for the quantification of SFX and its TPs

The performance of the method was evaluated by measuring quality parameters as linearity, recovery, repeatability and sensitivity for each of the target compounds. Mass parameters established for the MRM acquisition mode were optimized for each target compound as indicated in Table 3.1. The linear calibration curve was generated using linear regression analysis over the concentration range from 0.1 to 100 μg/L, where good fit was achieved ($r^2 > 0.99$) for all the analytes.

The recoveries of the extraction step in effluent samples were determined in triplicate by comparing the concentrations obtained after the SPE procedure with the initial spiking concentration (400 ng/L), both calculated by internal standard calibration. Since effluents samples contain already target compounds, non-spiked effluents samples were also analyzed and the levels found were subtracted from those obtained from spiked samples. The recoveries obtained were 89% for SFX, 62% for 4-NO_2-SFX, 104% for Desamino-SFX and 109% for N-Acetyl-SFX. The relative standard deviation between three replicates was always below 15%, indicating good repeatability.

The sensitivity of the method was established by measuring the method detection limits (MDL) and method quantification limits (MQL), both determined in spiked samples (n=3) as the minimum detectable amount of analyte with signal-to-noise ratio of 3 for MDL and 10 for MQL.

The methodology applied to samples analyzed by direct injection provides MDL of 4.67 ng/L for SFX, 37.96 ng/L for 4-NO_2-SFX, 7.41 ng/L for Desamino-SFX and 11.26 ng/L for N^4-Acetyl-SFX whereas MQL reached were 15.60 ng/L, 93.20 ng/L, 24.81 ng/L and 37.53 ng/L, respectively. The MDL achieved for samples preconcentrated (effluent samples) were 0.59 ng/L for SFX, 4.05 ng/L for 4-NO_2-SFX, 1.05 for Desamino-SFX and 2.73 ng/L for N^4-Acetyl-SFX whereas MQL were 1.95 ng/L, 13.73 ng/L, 4.22 ng/L and 10.94 ng/L, respectively. The relative standard deviation between three replicates was always below 15%, indicating good repeatability.

Chapter II

Unraveling the potential of a combined nitritation-anammox biomass towards the biodegradation of pharmaceutically active compounds

Section 5.3.1: Activity tests

Composition of bacterial communities in the pilot scale combined N/A reactor

Suspended and attached bacterial communities showed clear differences in the relative abundance of several bacterial taxa such as Bacteroidetes, Chlorobi, Chloroflexi, Alpha- and Betaproteobacteria (Figure S14a). Interestingly, sequences affiliated to families *Saprospiraceae* (phylum Bacteroidetes) and *Nitrosomonadaceae* (class Betaproteobacteria) were prevalent in the liquid phase whereas attached communities showed a more evenly distribution of bacterial taxa. In carriers, bacterial communities were more enriched in sequences affiliated to uncultured Chloroflexi and family SJA-28 within the phylum Chlorobi.

Interestingly, AOB (*i.e.* genus *Nitrosomonas*) were prevalent in the liquid phase whereas anammox bacteria (*i.e. Candidatus* Brocadia) and NOB (*i.e.* genus *Nitrospira*) were more enriched in the carrier-attached community (Figure S14b). In turn, the relative contribution of anammox bacteria to the overall ammonia-oxidizing community in the suspended fraction was one order of magnitude lower than that found in the carriers (2% and 28%, respectively; Figure S14b). Comparison of the Shannon index of diversity confirmed that the bacterial community in the carriers was significantly more diverse than that in the liquid phase (Shannon index of 6.43±0.07 and 5.54±0.02, respectively, p=0.0065). However, no significant differences were obtained when communities were compared on the basis of their phylogenetic similarity using the weighted UniFrac distance (PERMANOVA, p=0.342, (data not shown).

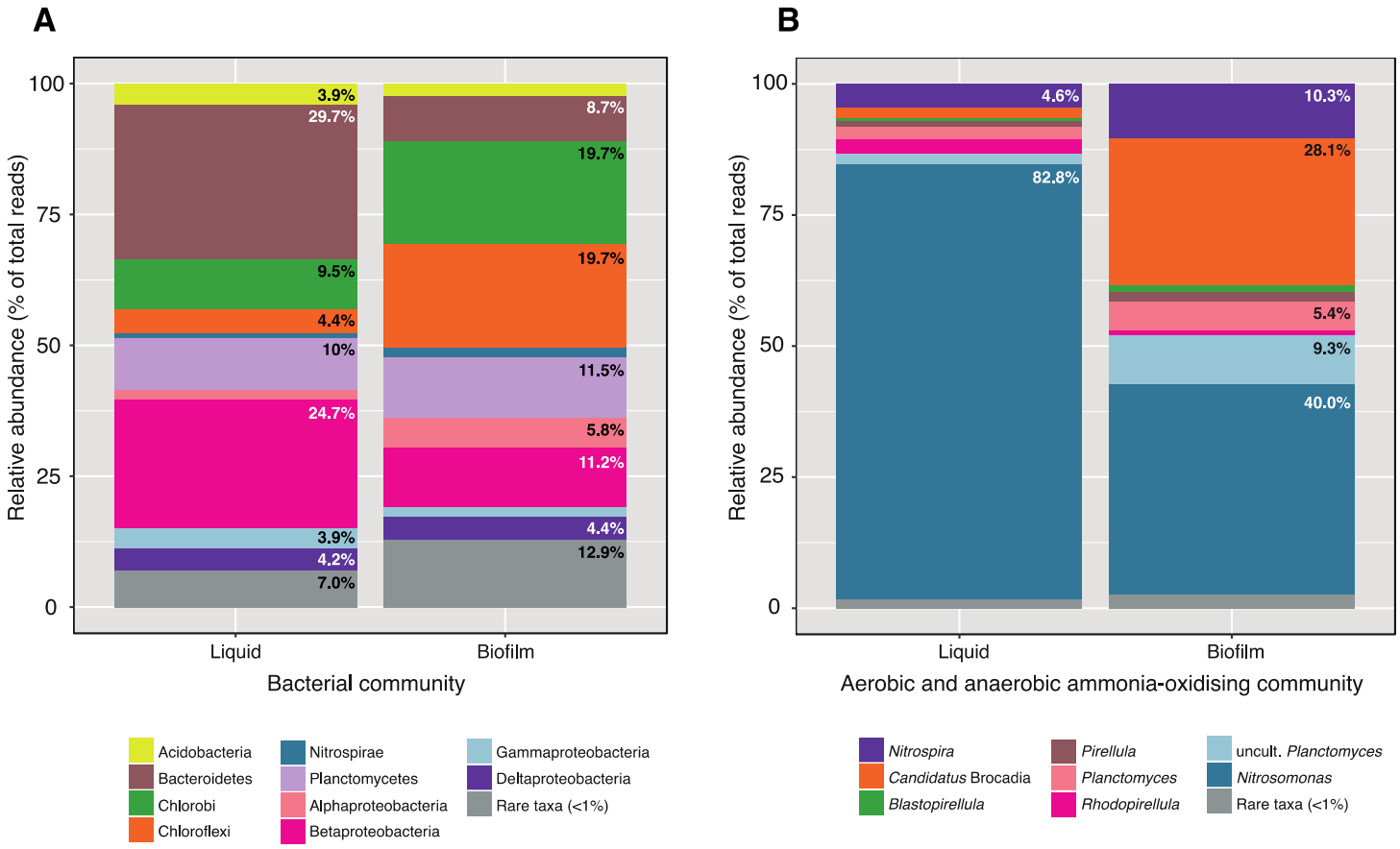


Figure S14: Average relative composition of bacterial communities inside the biological 400 L pilot-scale reactor. a) Composition of the overall bacterial community at phylum level (class for Proteobacteria), and b) Composition of the aerobic and anaerobic ammonia-oxidizing community at genus levels. Values are the mean of biological duplicates after normalizing samples to the same number of reads (29,000).

Section 5.3.2: Batch experiments

A time course of IBU and its detected TPs (i.e. 1-hydroxyl-IBU and 2-hydroxyl-IBU) for the different experimental conditions applied, are presented in Figure S15.

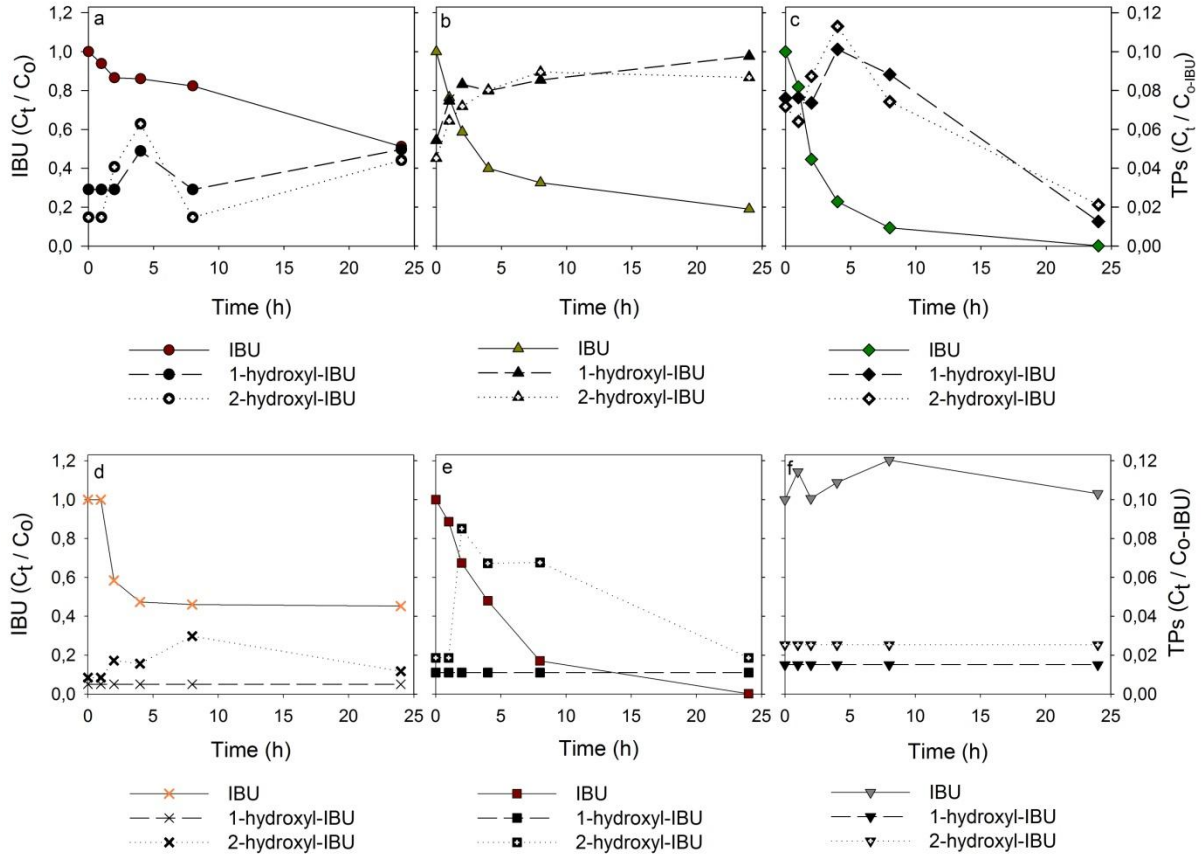


Figure S15: Removal of IBU and TPs formation under the studied experimental conditions: a) combined N/A, b) Aerobic-NB, c) aerobic-ATU, d) anoxic-ANX, e) Aerobic-HET and f) Anoxic-HET.

Chapter III

Comparative assessment of endocrine disrupting compounds removal in heterotrophic and enriched nitrifying biomass

Section 6.3.1: EDCs' elimination at different SAORs

Table S3: SAORs obtained during the experiments with NAS, AOB and HET

	Name	Code	SAOR
NAS	Different NH_4^+ & SAORs	T1	0.49
		T2	0.41
		T3	0.41
		T4	0.28
		T5	0.16
		T6	0.14
		T7	0
		T8	0
	NAS + ATU	T9	0
		T10	0
AOB	Different NH_4^+ & SAORs	T11	0.54
		T12	0.47
		T13	0.42
		T14	0.35
		T15	0.13
		T16	0.12
		T17	0
		T18	0
	AOB + ATU	T19	0
		T20	0
HET	Only EDCs	T21	--
		T22	--
	NH_4^+	T23	0.09
		T24	0.03
	ACE	T25	0
		T26	0
	NH_4^+ + ACE	T27	0.03
		T28	0.02
	NH_4^+ + ACE + ATU	T29	0