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Understanding the enzymatic and cometabolic biotransformation of organic micropollutants under aerobic heterotrophic conditions

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TESIS DE DOCTORADO

Understanding the enzymatic and cometabolic biotransformation of organic micropollutants under aerobic heterotrophic conditions

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Understanding the enzymatic and cometabolic biotransformation of organic micropollutants under aerobic heterotrophic conditions

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En Santiago de Compostela, 21 de diciembre de 2021

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ABSTRACT

The consumption of organic micropollutants (OMPs) such as pharmaceuticals, personal care products, hormones and industrial chemicals has increased globally in the past decades. A large fraction of these compounds end up in wastewater treatment plants (WWTPs), which have not been designed to eliminate them and serve as their gateway to the aquatic environment. Therefore, very low concentrations (in the ng-µg L^{-1} range) of OMPs reach surface, ground and even drinking water, raising an emerging concern due to their potential to harm the environment and human health.

The most common biological treatment in WWTPs is based on an activated sludge system, which typically involves heterotrophic and autotrophic nitrifying activities, favoring the removal of OMPs thanks to their combined action. However, the specific role that each population plays is still largely unknown, particularly in the case of the heterotrophs, which are gaining increased relevance in the design of novel, more energy-efficient WWTPs.

To maximize OMPs removal in WWTPs and minimize their environmental impact, in-depth knowledge about their biotransformation mechanisms is essential. Therefore, the goal of this PhD thesis is to increase the understanding of the biotransformation processes occurring under aerobic heterotrophic conditions. The focus is placed on *(i)* analyzing the influence of the heterotrophic activity on the biotransformation of OMPs; *(ii)* determining whether biotransformation happens cometabolically when OMPs are present at environmental concentrations; *(iii)* identifying the main biotransformation reactions catalyzed by aerobic heterotrophs; *(iv)* deciphering the heterotrophic microbial and enzymatic key players involved in OMPs biotransformation. To that end, multiple experiments in continuous, sequential batch and batch reactors were carried out and OMPs biotransformation, transformation product (TP) formation, metagenomic and metaproteomic analyses were performed.

The results obtained highlight that aerobic heterotrophs can efficiently and extensively biotransform OMPs presenting a wide range of physicochemical properties. In fact, the heterotrophic biotransformation extent observed was similar to literature reported values of activated sludge or purely nitrifying systems, suggesting that in some cases there may not be the need to maintain the nitrifying activity in WWTPs to reduce the presence of OMPs. Such biotransformation occurred simultaneously with the biodegradation of the primary carbon source and the specific biotransformation rate was

directly influenced by the organic loading rate, proving cometabolism as the main responsible biotransformation mechanism. Besides, the increases in the biotransformation rate with higher heterotrophic activities happened thanks to variations in the biotransformation kinetic constant (k_{biol}), highlighting that k_{biol} is not only dependent on OMPs physicochemical properties and environmental conditions, but also on the specific biomass activity, which is driven by the primary substrate.

In the experiments, multiple TPs were confidently identified, emphasizing that the quantification of OMPs removal alone is not sufficient and that biotransformation pathways and TPs formation need to be elucidated to properly analyze the environmental risks caused by OMPs. The main transformation reactions observed to take place under heterotrophic conditions were: hydrolysis, conjugation and oxidation, which was subdivided into hydroxylation, deamination, demethylation and dehydrogenation. Such reactions were linked to oxygenase, dehydrogenase, hydrolase and transferase enzymatic activities and, for some compounds, several TPs were observed, pointing towards sequences of transformation steps or transformations taking place at different functional groups.

Additionally, further detailed experiments were performed with sulfamethoxazole (SMX), which is a highly relevant compound due to the increasing concern over the development of antibiotic-resistant genes and bacteria. Results confirmed that heterotrophs can extensively biotransform SMX while maintaining their capacity to biodegrade the primary carbon source and TPs analyses showed that 2,4(1H,3H)-pteridinedione-SMX (PtO-SMX), which is formed through the sulfonamide pterin-conjugation pathway, and N^4-acetyl-SMX are important metabolites of SMX under heterotrophic conditions. Besides, a clear shift in the heterotrophic bacterial community composition and protein expression related to the presence of SMX was observed through the application of genomic and metaproteomic techniques. Five bacterial families belonging to the phylum Proteobacteria showed characteristic trends (*Rhodobacteraceae, Comamonadaceae, Xanthobacteraceae, Devosiaceae* and *Hyphomicrobiaceae*) and the genus *Corynebacterium* and five enzymes involved in its central metabolism seemed to play a key role to mitigate SMX concentration.

Overall, the knowledge acquired in this thesis helps to better understand cometabolic biotransformation processes taking place under aerobic heterotrophic conditions. This

insight can be extremely helpful in the design and optimization of WWTPs, aiming to reduce OMPs concentration and the risks related to their presence in the environment.

Keywords: cometabolism; enzymes; heterotrophs; omics; pharmaceuticals; transformation products; wastewater

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LIST OF ACRONYMS

ADBI: Celestolide

AEBSF: 4-benzenesulfonyl fluoride hydrochloride

AHTN: Tonalide

AMO: Ammonia monooxygenase

AOA: Ammonia oxidizing archaea

AOB: Ammonia oxidizing bacteria

ATU: Allylthiourea

BES: 2-bromoethanesulfonate

BPA: Bisphenol A

CBZ: Carbamazepine

COD: Chemical oxygen demand

DCF: Diclofenac

DZP: Diazepam

E1: Estrone

E2: 17β-estradiol

EE2: 17α-ethinylestradiol

ERY: Erythromycin

FAS: Ferrous ammonium sulphate

FLX: Fluoxetine

HHCB: Galaxolide

HRT: Hydraulic retention time

IBP: Ibuprofen

LOQ: Limit of quantification

NED: N-(1-naphthyl)ethylenediamine

NOB: Nitrite-oxidizing bacteria

NP: 4-nonylphenol

NPX: Naproxen

OCT: Octyne

OLR: Organic loading rate

OMP: Organic micropollutant

OP: 4-octylphenol

ROX: Roxithromycin

SPE: Solid phase extraction

SMX: Sulfamethoxazole

SRT: Sludge retention time

TCA: Tricarboxylic acid

TCS: Triclosan

TMP: Trimethoprim

TP: Transformation product

TSS: Total suspended solids

USE: Ultrasonic solvent extraction

VSS: Volatile suspended solids

WWTP: Wastewater treatment plant

RESUMO

Capítulo 1. Introdución e contexto

Os microcontaminantes orgánicos (MCOs) representan unha extensa gama de sustancias de consumo crecente en todo o mundo que inclúe produtos farmacéuticos, de uso persoal e hormonas, entre outros. Estes compostos, a pesar de estar presentes en concentracións moi baixas, no rango de ng a µg L^{-1}, supoñen un risco tanto para o medioambiente como para a saúde humana a causa da súa persistencia, bioacumulación e actividade biolóxica. Ademais, poden acentuar fenómenos de toxicidade, mutaxenicidade, resistencia antibiótica e estres oxidativo, así como causar desordes dos sistemas nervioso, hormonal e reprodutivo.

Os MCOs rematan nas estacións depuradoras de augas residuais (EDAR), que os reciben a través de diferentes rutas, como nas augas residuais urbanas, hospitalarias e industriais. Non obstante, a pesar de ser capaces de reducir as concentracións de moitos compostos, as EDAR non foron deseñadas para eliminar os MCOs, que frecuentemente son emitidos ao medioambiente sen tratar. Ademais, as aplicacións, propiedades físico-químicas e persistencia dos MCOs é moi ampla e as condicións medioambientais e de proceso aplicadas (como temperatura, pH, comunidade microbiana ou potencial redox, entre outros) son fundamentais para reducir a súa concentración, o que da lugar a grandes diferenzas na súa presenza e eficiencia de eliminación nas EDAR.

A eliminación dos MCOs nos procesos biolóxicos das EDAR ocorre principalmente por *(i)* volatilización, *(ii)* sorción ao lodo e *(iii)* biotransformación, aínda que para algúns compostos tamén se poden producir procesos de transformación abiótica. A etapa de biotransformación está considerada como o mecanismo principal xa que a volatilización e a sorción soamente son relevantes para compostos que presentan altos valores da constante da lei de Henry e do coeficiente de partición sólido-líquido, respectivamente.

Dita biotransformación pode producirse metabólicamente se a enerxía xerada a partir da reacción de transformación dos MCOs é suficiente para promover o crecemento e mantemento celular. Sen embargo, a causa das baixas concentracións ás que están presentes os MCOs nas EDAR, a biotransformación xeralmente se produce cometabólicamente. Durante o cometabolismo, os microorganismos transforman compostos que non son de crecemento e non poden ser utilizados como única fonte de nutrientes e enerxía na presencia obrigada dun substrato primario. Este último atópase a maiores concentracións no medio, serve como doador de electróns e pode manter a

poboación microbiana e inducir a produción de enzimas e cofactores que son capaces de biotransformar os MCOs grazas a súa versátil actividade catalítica e a similitudes entre a estrutura química dos MCOs e a dos substratos naturais da enzima.

Os procesos de biotransformación frecuentemente dan lugar á mineralización incompleta dos MCOs, que son convertidos en produtos de transformación (PT) que poden posuír os mesmos ou incluso maiores efectos adversos para o ecosistema que os seus compostos de orixe. Deste xeito, para reducir os riscos da descarga dos MCOs ao medioambiente e para maximizar a súa eliminación, é fundamental comprender os mecanismos de biotransformación que ocorren nos compartimentos biolóxicos das EDAR. Este estudo debe prestar especial atención aos PTs formados e buscar identificar os microorganismos e actividades enzimáticas que xogan un rol principal no proceso de biotransformación.

O tratamento biolóxico máis común nas EDAR baséase nun sistema de lodos activados, que normalmente implica actividades heterótrofas e autótrofas nitrificantes. Mentres que a segunda foi amplamente estudada a causa do relevante rol xogado pola enzima amonio monooxixenasa, o papel dos microorganismos aerobios heterótrofos na biotransformación dos MCOs aínda non está claro a causa da complexidade e variedade das súas actividades metabólicas. O estudo dos mecanismos heterótrofos de biotransformación é importante pola relevancia desta poboación para eliminar a materia orgánica e polo seu crecente interese en novas concepcións de EDAR enerxéticamente máis eficientes, onde as etapas A, que traballan a altas taxas de carga orgánica e curtos tempos de retención de sólidos, operan basicamente en condicións heterótrofas.

Polo tanto, esta tese busca obter una mellor imaxe dos mecanismos que dan lugar a biotransformación cometabólica dos MCOs baixo condicións aerobias heterótrofas, intentando responder as seguintes cuestións:

- Son capaces os organismos heterótrofos de biotransformar amplamente MCOs con diversas propiedades físico-químicas?
- Prodúcese cometabólicamente dita biotransformación?
- Unha maior actividade heterótrofa afecta a eficiencia de biotransformación dos MCOs? E a súa cinética ou constante cinética de biotransformación?

- Cales son as principais reaccións de biotransformación que levan a cabo os organismos heterótrofos?
- Existe algunha poboación microbiana heterótrofa que xogue un papel destacado durante a biotransformación dos MCOs?
- Cales son as actividades enzimáticas máis relevantes durante a biotransformación cometabólica dos MCOs en condicións aerobias heterótrofas?

Capítulo 2. Materiais, métodos e equipos

Neste capítulo descríbese a alimentación utilizada para o desenvolvemento dos experimentos levados a cabo nos distintos tipos de reactor (en continuo, secuenciais en batch e en batch). Ademais, preséntanse os MCOs estudados ao longo da tese e os métodos analíticos utilizados para a súa determinación e a dos PTs formados. Adicionalmente, proporcionase unha descrición detallada dos métodos analíticos empregados para monitorizar o estado dos experimentos (demanda química de oxíxeno (DQO), especies de nitróxeno, concentración de sólidos, oxíxeno disolto, pH e temperatura) e para obter información microbiolóxica (ADN e proteínas), que serviu para identificar os microorganismos e enzimas clave involucrados na biotransformación dos MCOs. Finalmente, preséntanse os cálculos empregados para analizar os datos obtidos nos distintos capítulos.

Capítulo 3. A velocidade de carga orgánica afecta a cinética de biotransformación cometabólica baixo condicións heterótrofas en sistemas de lodos activos

O rol dos microorganismos aerobios heterótrofos durante a biotransformación dos MCOs nos sistemas de lodos activos aínda non está claro. Neste capítulo, búscase dar resposta a súa capacidade para biotransformar cometabólicamente un grupo de 15 MCOs con diversas propiedades físico-químicas engadidos en concentracións entre 1 e 40 µg L^{-1}, así como coñecer a influenza da actividade heterótrofa na cinética de biotransformación cometabólica.

Con este obxectivo, operouse un reactor continuo de tanque axitado (CSTR) a catro velocidades de carga orgánica (VCO) diferentes (0.2, 0.4, 0.6 e 0.8 g DQO L^{-1} d^{-1}) a través do uso de concentracións crecentes de substrato primario (acetato) e mantendo constante

o tempo de retención hidráulico (24 h). Adicionalmente, para asegurar unicamente a presenza de actividade heterótrofa, engadiuse aliltiourea, un inhibidor nitrificante, e mantívose o tempo de retención celular ao redor de 5 días. Ademais, usando como inoculo os respectivos reactores CSTR, tamén se levaron a cabo ensaios en batch con concentracións iniciais de substrato primario (acetato) de 0.2, 0.4, 0.6 e 0.8 g DQO L^{-1}. O obxectivo destes ensaios foi observar se variacións na actividade heterótrofa microbiana, determinada como a actividade específica máxima de DQO, afectaban a cinética de biotransformación dos MCOs e, particularmente, a constante de biotransformación cinética (k_{biol}).

En primeiro lugar, os resultados dos CSTR amosaron que a eficacia de biotransformación foi superior ao 80 % para a maioría dos MCOs, coa excepción de carbamazepina e diazepam (inferior a 20 % a causa da súa natureza recalcitrante), trimetoprim (35 %) e eritromicina (70 %), e que os valores se mantiveron constantes ás 4 VCOs estudadas. Estes datos confirman as altas capacidades dos microorganismos heterótrofos para biotransformar os MCOs. Ademais, observouse que a biodegradación da fonte de carbono primaria e a biotransformación dos MCOs ocorría de modo simultáneo e que maiores VCOs deron lugar a maiores velocidades específicas de biotransformación, destacando ao cometabolismo como mecanismo principal de biotransformación baixo as condicións estudadas.

Por outra banda, os ensaios en batch permitiron observar que a maior tasa de biotransformación específica determinada nos reactores CSTR a maiores actividades heterótrofas ocorre a través de variacións en k_{biol}. Este resultado permite afirmar que a k_{biol} dun determinado composto non depende soamente das súas propiedades físicoquímicas e das condicións ambientais, senon tamén da actividade específica do lodo causada polo substrato primario. A correlación positiva entre k_{biol} e a actividade específica máxima de DQO foi observada para todos os MCOs non recalcitrantes, con excepción de dúas fragancias. Non obstante, a influenza da actividade heterótrofa foi desigual entre os compostos estudados, indicando que a súa acción é específica para cada MCO.

En conclusión, neste capítulo amósase que maiores VCOs conducen a maiores tasas de biotransformación cometabólica dos MCOs a través do aumento dos seus respectivos valores de k_{biol}. Non obstante, isto non implica necesariamente unha mellora no rendemento de biotransformación dos compostos sempre que se aporte un tempo de

retención hidráulico suficiente. Así, a variación da carga orgánica da EDAR, a xestión dos substratos orgánicos e o control da actividade microbiana aparecen como parámetros fundamentais que rexen a biotransformación dos MCOs.

Capítulo 4. Biotransformacións enzimáticas heterótrofas de microcontaminantes orgánicos en sistemas de lodos activos

Os organismos aerobios heterótrofos constitúen a maior fracción da biomasa nos sistemas de lodos activos e teñen a capacidade para contribuír notablemente a biotransformación dos MCOs, como se amosa no capítulo 3. Non obstante, os mecanismos de biotransformación e as enzimas presentes no seu metabolismo encargadas de catalizar ditas reaccións aínda non foron claramente determinadas pola falta de información sobre os procesos enzimáticos e a complexidade dos procesos biolóxicos. Dito coñecemento é fundamental para poder implementar estratexias nas EDAR que faciliten o desenvolvemento dos microorganismos e enzimas clave que maximicen a eliminación dos MCOs.

Neste capítulo operouse un reactor CSTR nas mesmas condicións ás descritas no Capítulo 3 e ao que se engadiron 20 MCOs en concentracións entre 1 e 40 μg L^{-1}. Mediante técnicas de cromatografía líquida con espectrometría de masas en tándem (LC-MS MS), realizáronse análises para detectar posibles PTs dos MCOs. Dita información foi utilizada para identificar as principais reaccións de biotransformación catalizadas polos organismos heterótrofos e para predicir posibles actividades enzimáticas responsables de catalizar ditas reaccións.

Os resultados, en liña có observado no Capítulo 3, amosaron unha elevada eliminación para a maioría dos compostos (por riba do 75 % para todos os MCOs agás no caso da carbamazepina, diazepam e trimetoprim), principalmente a causa de biotransformación, aínda que tamén se observou unha sorción relevante ao lodo no caso da fluoxetina e as fragancias utilizadas. Comparando a biotransformación alcanzada pola poboación heterótrofa presente no reactor con valores reportados en literatura para sistemas nitrificantes ou de lodos activos que presentan actividades heterótrofa e nitrificante, observouse que se alcanzaron eficacias de biotransformación similares, cuestionando a necesidade de manter a actividade nitrificante nas EDAR para reducir a presenza destes MCOs.

Por outra banda, tamén se identificaron con elevada confianza 12 PTs para os 20 MCOs investigados. No caso de celestolide e da fluoxetina, observáronse dous PTs diferentes, apuntando a secuencias de biotransformación que teñen lugar en diferentes grupos funcionais. Os PTs foron asignados a tres tipos de reaccións de biotransformación: oxidación, hidrólise e conxugación. As reaccións de oxidación, que son frecuentemente observadas como paso de biotransformación inicial para moitos MCOs en sistemas de lodos activos, foron subdivididas en reaccións de hidroxilación, desaminación, desmetilación e deshidroxenación. A partir dun detallado análise das reaccións e das estructuras dos compostos, puideron predicirse as probables actividades enzimáticas presentes no metabolismo heterótrofo encargadas de catalizar a formación dos 12 PTs atopados. Ditas enzimas pertencen ás clases das oxixenasas (subdivididas en monooxixenasas e dioxixenasas), deshidroxenasas, hidrolasas e transferasas.

Capítulo 5. O sulfametoxazol desencadea actividades enzimáticas específicas en condicións aerobias heterótrofas: un enfoque metaproteómico

Os antibióticos representan un grupo destacado de MCOs que cada vez se detectan con maior concentración nas EDAR debido ao seu uso crecente nas sociedades modernas. Como consecuencia, nos últimos tempos as EDAR estanse convertendo en lugares clave para o desenvolvemento de xenes e bacterias resistentes aos antibióticos, o que supón un gran risco medioambiental e para a saúde humana. Dentro dos diferentes antibióticos, as sulfonamidas teñen particular relevancia pola súa extensa produción e consumo a nivel global nos ámbitos médico e veterinario. Dentro deste grupo, o sulfametoxazol (SMX) é o antibiótico máis utilizado e é frecuentemente detectado nos solos e na auga. A causa da crecente preocupación que supón a súa presenza nas EDAR e descarga ao medioambiente, recentemente foi incluído na "Lista de vixilancia das augas superficiais" publicada pola Unión Europea có obxectivo de supervisar e recoller datos sobre os seus riscos potenciais para o medio acuático. Polo tanto, neste capítulo búscase afondar nos mecanismos aerobios heterótrofos de biotransformación do SMX e descifrar as principais actividades enzimáticas e microorganismos involucrados no proceso.

O deseño experimental consistiu na operación de reactores en continuo e secuenciais en batch alimentados cos mesmos macro e micronutrientes utilizados nos capítulos 3 e 4.

Por unha banda, o reactor en continuo operouse durante 5 meses, usando como substrato primario o acetato, asegurando a ausencia de nitrificación e fixando o TRH e TRC en 1 e 5 d, respectivamente. Ademais, o período experimental dividiuse en tres etapas: (i) fase de control sen SMX; (ii) SMX engadido en 50 µg L^{-1} e (iii) SMX engadido en 1000 µg L^{-1}. Ao final de cada etapa colléronse mostras para levar a cabo análises de biotransformación, PTs e xenómica. Por outra banda, 18 reactores secuenciais en batch operáronse durante 25 días e dividíronse en 6 grupos en triplicado aos que se engadiron as seguintes concentracións de SMX: 0 (control), 50, 250, 500, 1000 e 2000 µg L^{-1}. Durante o desenvolvemento do experimento colléronse mostras para levar a cabo análises de PTs, metaxenómica e metaproteómica.

No reactor operado en continuo os resultados amosaron que a presenza de SMX non afectou á eliminación do substrato primario (acetato) e observouse unha biotransformación no rango do 70-80 % para o SMX. Además, detectáronse dous PTs: 2,4(1H,3H)-pteridinedione-SMX (PtO-SMX) e N^4-acetyl-SMX. Adicionalmente, os resultados xenómicos amosaron un claro cambio na composición da comunidade bacteriana relacionado coa presenza de SMX e a súa concentración inicial na alimentación. Concretamente, destacaron as tendencias características seguidas por cinco familias bacterianas (*Rhodobacteraceae, Comamonadaceae, Xanthobacteraceae, Devosiaceae* e *Hyphomicrobiaceae*) pertencentes ao filo bacteriano Proteobacteria.

Nos reactores secuenciais en batch os resultados amosaron que a presenza do SMX nas concentracións utilizadas tampouco afectou negativamente a eliminación do substrato primario. Ademais, houbo unha elevada biotransformación do SMX, aínda que se observaron dúas tendencias características. Por unha parte, a biotransformación en tódolos reactores aumentou entre o día 2 e o 17, seguramente grazas a eventos de adaptación microbiana, e despois no día 25 descendeu, posiblemente por toxicidade a causa da acumulación de PTs no medio. Por outra banda, a eficiencia de biotransformación foi en xeral menor nos experimentos con maiores concentracións de SMX, o que tamén podería ser debido a unha maior acumulación de PTs.

Durante o experimento, e ao igual que no reactor operado en continuo, identificouse o TP PtO-SMX, que xa foi previamente detectado nas EDAR e que se forma a partir dunha ruta de conxugación das sulfonamidas cando estas interaccionan coa enzima dihidropteroato sintetasa, impedindo a síntese de ácido fólico. Non obstante, as enzimas

involucradas na formación dos PTs desta ruta non puideron ser identificadas, posiblemente pola súa baixa abundancia.

Adicionalmente, a través dos análises metaxenómicos e metaproteómicos identificouse o xénero *Corynebacterium* como fundamental na biotransformación do SMX e cinco enzimas relacionadas co seu metabolismo central aumentaron a súa abundacia a maiores concentracións do antibiótico. Polo tanto, os resultados suxiren que *Corynebacterium* podería estar relacionada co consumo de SMX como fonte de nutrientes e que simultaneamente o microbioma podería desenvolver unha ruta de biotransformación cometabólica co SMX, dando lugar a formación de PtO-SMX.

En conclusión, parece posible a coexistencia dos mecanismos metabólicos e cometabólicos de biotransformación de SMX, posiblemente debido á acción de bacterias de distintos xéneros. Ademais, é destacable que determinadas enzimas involucradas en tareas do metabolismo central poderían participar na biotransformación do SMX e se demostra que a metaproteómica pode ser unha ferramenta útil para desvelar os microorganismos e enzimas clave que participan na biotransformación dos MCOs.

Capítulo 6. Discusión xeral e conclusións

Neste capítulo intégranse e discútense conxuntamente os resultados obtidos durante o desenvolvemento da tese, buscando responder as preguntas expostas na introdución acerca da biotransformación de MCOs en condicións aerobias heterótrofas. **Son capaces os microorganismos heterótrofos de biotransformar os MCOs? A biotransformación é cometabólica? Cales son as principais reaccións de biotransformación? Que microorganismos e enzimas catalizan dito proceso?**

O coñecemento xerado durante o desenvolvemento desta tese busca facilitar o deseño e aplicación de estratexias nas EDAR que permitan reducir a concentración dos MCOs e a súa emisión ao medioambiente. O papel dos microorganismos aerobios heterótrofos nas EDAR durante a biotransformación dos MCOs pode ser moi relevante, grazas a súa elevada capacidade para eliminar numerosos compostos (**Capítulo 3**). Esta biotransformación, cando os MCOs están presentes a baixas concentracións, ocorre de modo cometabólico e a actividade heterótrofa permite regular a velocidade específica de biotransformación dos compostos a través de variacións na súa constante cinética de biotransformación (k_{biol}), potencialmente facilitando operar nas EDAR a tempos de

retención hidráulico máis baixos. Non obstante, unha maior actividade heterótrofa pode non dar lugar a unha eficacia maior biotransformación sempre que o tempo de retención hidraúlico sexa suficientemente elevado, posiblemente a causa de limitacións termodinámicas que poñen un límite a eficacia de biotransformación.

Durante a biotransformación heterótrofa dos MCOs prodúcese a formación de PTs (**Capítulo 4 e 5**). O seu análise, ademais de ser relevante para coñecer a súa ecotoxicoloxía, permite obter información sobre as reaccións de biotransformación que leva a cabo unha determinada poboación microbiana e, polo tanto, posibilita intentar identificar as enzimas clave que catalizan ditas reaccións de biotransformación. Nesta tese observouse que os microorganismos heterótrofos levan a cabo fundamentalmente reaccións de oxidación, hidrólise e conxugación, fundamentalmente catalizadas por oxixenasas, deshidroxenasas, hidrolasas e transferasas.

A aplicación de técnicas omicas recentemente está gañando relevancia para identificar biomarcadores clave na biotransformación dos MCOs. Este enfoque parece moi prometedor porque permite caracterizar o conxunto completo de xenes ou produtos xenéticos presentes nun microbioma nun momento concreto, pero aínda existen dúbidas sobre a súa aplicación a causa da fiabilidade para identificar os microorganismos e enzimas cando os MCOs están presentes en concentracións moi baixas e porque pode xerar un gran número de falsos positivos. Nesta tese, mediante metaxenética e metaproteómica, determináronse cambios na composición da comunidade bacteriana heterótrofa en relación coa presenza de SMX e se suxire o rol destacado do xénero *Corynebacterium* e de varias enzimas presentes no seu metabolismo central durante a biotransformación do SMX (**Capítulo 5**). Polo tanto, estes resultados indican que as técnicas omicas poden ser unha ferramenta moi útil para determinar relacións causais entre a biotransformación dos MCOs e os microorganismos e enzimas clave.

Finalmente, se expoñen distintas posibilidades de investigación futura e diversos retos que aínda quedan pendentes para comprender mellor os procesos de biotransformación dos MCOs nas EDAR. Destaca a importancia de aplicar múltiples técnicas durante o procedemento experimental para unha identificación fiable das actividades enzimáticas e os microorganismos involucrados na biotransformación dos MCOs, incluindo o análise de PTs, os estudos omicos ou os ensaios *in vitro*. Así mesmo, as principais liñas de investigación futura que se propoñen son: aquelas que intenten encontrar a fronteira entre o cometabolismo e o metabolismo; descifrar o rol de

actividades secundarias presentes na biomasa das EDAR; investigar as condicións medioambientais e operacionais que incrementan a expresión das enzimas clave; afondar en procedementos para extraer enzimas do lodo que permitan análises *in vitro*; desenvolver ferramentas analíticas máis sensibles e precisas dentro do ámbito omico e de PTs.

CHAPTER 1. INTRODUCTION AND CONTEXT

SUMMARY

Biotransformation of trace-level organic micropollutants (OMPs) by complex microbial communities in wastewater treatment facilities is a key process for their detoxification and environmental impact reduction. Therefore, understanding the metabolic activities and mechanisms that contribute to their biotransformation is essential when developing approaches aiming to minimize their discharge. This chapter addresses the relevance of cometabolic biotransformation and discusses the main enzymatic activities currently known to take part in the aerobic removal of OMPs in wastewater treatment plants. Furthermore, the most common methodologies to decipher such enzymes are discussed.

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Further information can be found in the “List of publications” (page 145).

1.1. ORGANIC MICROPOLLUTANTS IN WASTEWATER TREATMENT PLANTS

Organic micropollutants (OMPs) cover a wide range of substances of worldwide increasing consumption, including pharmaceuticals, personal care products, hormones and industrial chemicals (Luo et al., 2014). Despite being present at very low concentrations, in the ng to μg L^{-1} range, they pose the potential to harm the environment due to their persistence, bioaccumulation and biological activity (Margot et al., 2015). Besides, they can foster toxicity, mutagenicity, antibiotic resistance and oxidative stress, as well as causing disorders of the nervous, hormonal and reproductive system (Bilal et al., 2019; Tiwari et al., 2017). The applications, physicochemical properties and persistence of OMPs are very broad, causing their presence and removal efficiency to vary greatly in wastewater treatment plants (WWTPs), which, although they have proven to be capable of reducing their concentrations, were not designed to remove them (Margot et al., 2015; Petrie et al., 2014). The extent to which OMPs are removed also depends on environmental and process conditions, including temperature, pH, wastewater composition, microbial community and redox potential, among others (Alvarino et al., 2018a; Cao et al., 2020; Gulde et al., 2014; Kruglova et al., 2016; Rios-Miguel et al., 2021).

OMPs removal in biological processes of conventional WWTPs occurs mostly due to *(i)* volatilization, *(ii)* sorption to the sludge and *(iii)* biotransformation (Pomiès et al., 2013; Tiwari et al., 2017), although abiotic transformation processes may also take place for some compounds (Yu et al., 2018). Biotransformation is considered the main responsible mechanism since volatilization and sorption are only relevant for compounds showing high values of the Henry's law constant or solid-liquid partition coefficient, respectively (Nguyen et al., 2021; Su et al., 2021). OMPs biotransformation could occur metabolically if the energy generated from such reaction is sufficient to promote biomass growth and maintenance. Differently, cometabolic biotransformation is the ability of microorganisms to degrade non-growth substrates that cannot be used as the sole nutrient and energy source in the obligate presence of a primary substrate. The latter is available at higher concentrations, serves as an electron donor and can maintain the microbial population and induce the production of enzymes and cofactors which are capable of biotransforming the OMPs thanks to their versatile catalytic activity (Fernandez-Fontaina et al., 2014; Fischer and Majewsky, 2014; Krah et al., 2016). Among the scientific

community, it is widely assumed that the main biotransformation mechanism in real environmental conditions is cometabolism (Fischer and Majewsky, 2014; Lema and Suarez, 2017; Tran et al., 2013).

Either metabolically or cometabolically, biotransformation processes can lead to incomplete mineralization of OMPs, thus being converted into transformation products (TPs) that may pose even increased adverse effects to the aquatic ecosystems and human health (Gulde et al., 2016). Consequently, a thorough analysis of the fate and transformation reactions of the OMPs in the biological compartments of WWTPs is of vital importance to properly assess the environmental impacts (Men et al., 2017). For such reason, in recent times research has evolved from *(i)* trying to develop and optimize analytical methods to determine OMPs concentrations in different matrixes into *(ii)* assessing their occurrence in streams and compartments and determining the removal efficiencies of varying technologies and operational conditions and finally into *(iii)* attempting to comprehend the underlying removal mechanisms, metabolic pathways and enzymatic activities occurring in WWTPs during sorption and biotransformation processes (Alvarino et al., 2018a), which would greatly help to mitigate OMPs in WWTPs.

1.2. COMETABOLIC BIOTRANSFORMATION OF OMPS IN WWTPs

Previous studies have shown that many OMPs can be biotransformed metabolically or cometabolically (Fig. 1.1) depending on their physicochemical properties and the environmental conditions (Tran et al., 2013). Making a distinction between metabolism and cometabolism is important to have a better understating of the mechanisms involving OMPs biotransformation and to optimize their overall removal. However, multiple studies do not discriminate between them because differentiation can be difficult, particularly when working with mixed microbial communities such as activated sludge. Indeed, metabolism and cometabolism may coexist (Mandarić et al., 2018); sometimes is unclear if energy derives from OMPs biodegradation or dead cells (Tran et al., 2013); and metabolic and cometabolic steps can be interrelated and substitutable since they are part of a metabolic network evolved as a whole by the microbial community (Fischer and Majewsky, 2014). In this sense, microorganisms are continuously developing new catabolic pathways for substrates to access energy and nutrients or to detoxify compounds, and can develop new metabolic tools which may allow to turn cometabolic

processes into metabolic ones (Fischer and Majewsky, 2014; Kolvenbach et al., 2014). In fact, promiscuous enzymes, which can catalyze reactions with multiple substrates, have been associated with both processes that occur intentionally (metabolism) and fortuitously (cometabolism) (Fischer and Majewsky, 2014).

The metabolism of OMPs is challenging because growth-linked degradation reactions can only proceed when reaction thermodynamics and kinetics are favorable. To date, only heterotrophic microorganisms have proven to be capable of carrying out such process and, although the OMPs concentration threshold required for metabolic activities remains unclear, it appears to be at levels considerably higher than those typically detected in environmental samples and WWTPs (Nsenga Kumwimba and Meng, 2019; Tran et al., 2013). Thus, OMPs biodegradation may not be energetically favorable to microorganisms, requiring promiscuous enzymes to biotransform them thanks to structural similarities with primary substrates, even if such reactions tend to proceed at much lower rates (Kolvenbach et al., 2014; Lema and Suarez, 2017; Nguyen et al., 2021). As a consequence, in WWTPs, it is assumed that OMPs are biotransformed cometabolically as a side effect, linked to the elimination of macropollutants such as organic carbon, nitrogen and phosphorous (Fischer and Majewsky, 2014) and, perhaps, to endogenous decay when growth substrates are depleted (M. H. Kim et al., 2020). During cometabolism, the initial growth to non-growth substrate concentration ratio is important since high values may help microorganisms to recover from toxicity caused by OMPs and TPs and because initial substrate concentrations determine whether sufficient reducing power could be diverted to non-growth substrate biotransformation and cell growth and maintenance (M. H. Kim et al., 2020; Rios-Miguel et al., 2021). Accordingly, higher cometabolic biotransformation efficiencies could be expected with increased primary substrate loading rates, as observed in nitrifying and heterotrophic activated sludge (Fernandez-Fontaina et al., 2012; Helbling et al., 2012; Kennes-Veiga et al., 2020). However, it is not always the case since there is an upper (saturation limit) and lower (activation limit) threshold of primary substrate loading rates influencing cometabolism (Carneiro et al., 2020; Gonzalez-Gil et al., 2018b; Sheng et al., 2021). Loading rates below that threshold lead to insufficient reducing power supply for cometabolism and values above the threshold reach a biotransformation limit due to a cometabolic decoupling between OMPs and primary substrates (Sheng et al., 2021) or because of thermodynamic constraints such as chemical equilibrium or enzymatic reversibility

(Gonzalez-Gil et al., 2019a, 2018a, 2018b). Interestingly, some studies have also observed that under starvation conditions or low availability of easily degradable carbon, biotransformation may improve thanks to the stimulation of multiple microorganisms and an increase in the abundance or diversity of some enzymes (Achermann et al., 2018b; Nguyen et al., 2021). The specificity constant ratio, or kinetic efficiency, of non-growth to growth substrates of a microbial culture, which characterizes the inherent competition, is also key during cometabolism since competition for the enzymatic active site may occur, leading to hindered biotransformation of some OMPs due to the higher concentrations at which growth substrates are present (Gonzalez-Gil et al., 2021; M. H. Kim et al., 2020; Plósz et al., 2010). Non-competitive inhibition may also occur when OMPs and primary substrates are not structurally analogous and bind to different active sites, resulting in reduced overall oxidation rates (Lema and Suarez, 2017; Su et al., 2015). Furthermore, the presence of specific moieties in OMPs may facilitate or hinder cometabolism and influence initial biotransformation reactions that condition the likelihood of subsequent reaction steps and the overall biotransformation rate and efficiency (Helbling et al., 2012; Kolvenbach et al., 2014; Nguyen et al., 2021; Tran et al., 2013).

OMPs cometabolism has been observed in multiple environments. For instance, under nitrifying conditions, it was observed the cometabolic biotransformation of several OMPs along with ammonium oxidation (Gonzalez-Gil et al., 2021; Han et al., 2019; Sheng et al., 2021; Wang et al., 2019). Ammonia oxidizing bacteria (AOB) and archaea (AOA), the novel commamox population (Han et al., 2019) and slow-growing heterotrophs (Gonzalez-Gil et al., 2021) have been reported to participate in cometabolism, while other populations, such as nitrite-oxidizing bacteria (NOB), have shown a less relevant contribution (Yu et al., 2018). There is also evidence of cometabolism under heterotrophic conditions (Fernandez-Fontaina et al., 2016; Fischer and Majewsky, 2014; Gonzalez-Gil et al., 2021; Kennes-Veiga et al., 2020), although the carbon source of the primary substrate has a considerable influence on the biotransformation rate and extent (Larcher and Yargeau, 2011; Oliveira et al., 2019; Torresi et al., 2016a). The combination of nitrifying and heterotrophic activities has been shown to improve OMPs biotransformation and to reduce TPs accumulation thanks to the higher microbial diversity and a broader expression of non-specific enzymes (Fernandez-Fontaina et al., 2016; Khunjar et al., 2011). In contrast, information about cometabolism

under anoxic conditions is scarce despite evidence of their equal or even higher capabilities to biotransform some OMPs (Falås et al., 2013). Nonetheless, Torresi et al. (2017) proved cometabolism for several pharmaceuticals in denitrifying reactors and Martínez-Quintela et al. (2021), under the recently discovered N-Damo process, which consists of anaerobic methane oxidation with nitrite, also observed cometabolic biotransformation of multiple compounds. Similarly, anaerobic systems have also proven their capacity to reduce OMPs environmental impact in wastewater effluents (Arias et al., 2018; Phan et al., 2018) and it is believed that they could enhance the removal of some compounds poorly biotransformed under aerobic conditions (Harb et al., 2019; Lin et al., 2020). Gonzalez-Gil et al. (2017a, 2018b) and Carneiro et al. (2020) proved cometabolism for multiple compounds during anaerobic digestion; Alvarino et al. (2014) observed a linear relationship between the biotransformation rate of sulfamethoxazole, trimethoprim and naproxen and the methanogenic activity in an upflow anaerobic sludge blanket reactor and Oliveira et al. (2019) determined sulfamethazine cometabolism with anaerobic sludge using several carbon sources. Finally, OMPs cometabolism has also been observed in other biological processes, as in microalgae-based systems (Liu et al., 2021; Vo et al., 2020), in feast-famine conditions (Tang et al., 2021) and along with polyhydroxyalkanoates metabolism in a bioreactor set up for phosphorous removal (Torresi et al., 2019).

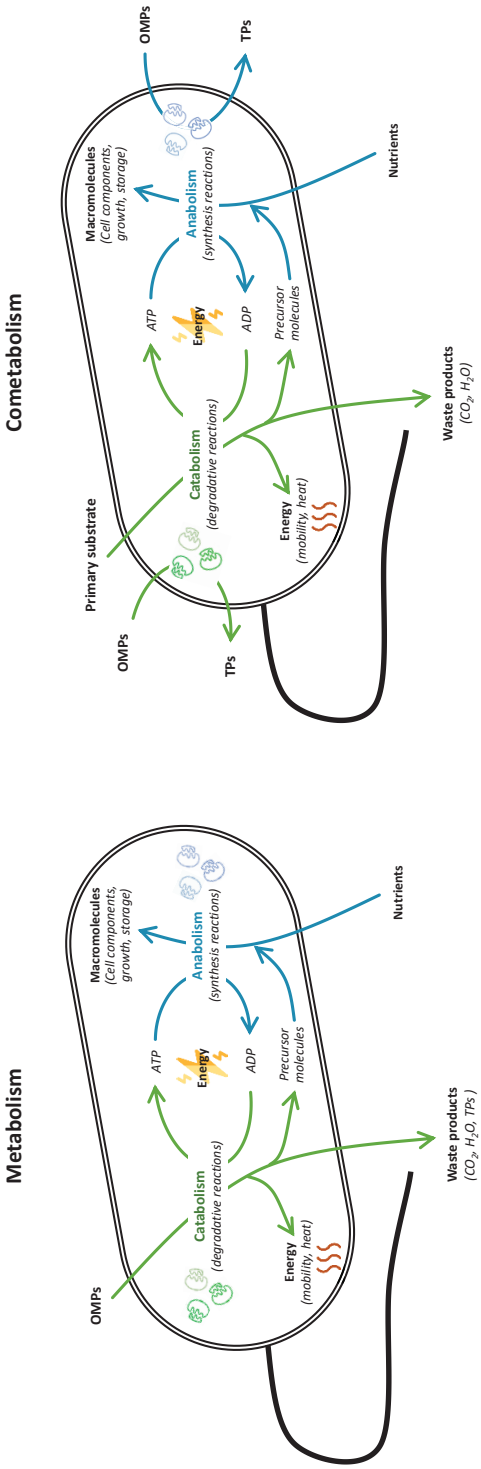

Fig. 1.1. Representation of the metabolic and cometabolic biotransformation of OMPs.

1.3. ENZYMATIC ACTIVITIES INVOLVED IN THE BIOTRANSFORMATION OF OMPs UNDER AEROBIC CONDITIONS

The metabolic capabilities and enzymatic network of the microbial communities present in WWTPs determine their biotransformation potential (Fischer and Majewsky, 2014; Helbling et al., 2012). However, the complexity of biological systems and the limited knowledge about enzymatic mechanisms has resulted in scarce information about the role of microbial strains and enzymes involved in biotransformation, which is crucial to develop risk-assessment tools that help predict OMPs pathways and half-lives in WWTPs (Achermann et al., 2020).

Cometabolic biotransformation consists of a broad sequence of individual reactions and several OMPs may undergo various removal strategies concurrently, leading to a huge array of candidate enzymatic activities that may participate in biotransformation (Helbling et al., 2010a; Kolvenbach et al., 2014). Such enzymes are frequently believed to be located intracellularly, carrying out most biotransformation reactions thanks to their high metabolic versatility. However, extracellular enzymes are particularly required for the break-down of large and ionized OMPs that pose difficulties for cellular uptake and limit biotransformation rates; and they may be highly relevant to reduce the selection pressure for antibiotic-resistant genes (Krah et al., 2016; Zumstein and Helbling, 2019). Similarly, it is generally assumed that biotransformation occurs thanks to catabolic enzymes, but anabolic enzymes also seem to play a key role, highlighting the challenge of deciphering them (Achermann et al., 2018a; Stadler et al., 2018).

The most common biological treatment in WWTPs is based on an activated sludge system where autotrophic nitrifiers and heterotrophs represent the most important microbial populations, although their relative contribution to OMPs biotransformation remains unclear (Lema and Suarez, 2017; Nguyen et al., 2021; Polesel et al., 2017). Nitrifying microorganisms can be classified into: AOB and AOA, involved in the oxidation of ammonia to nitrite, and NOB, capable of oxidizing nitrite to nitrate. Numerous studies performed in recent years have shown the key role played by nitrifying enzymes during biotransformation (Nsenga Kumwimba and Meng, 2019; Su et al., 2021) and, although further studies are needed to elucidate the role of promising enzymes such as hydroxylamine and nitrite oxidoreductases, enzymes present in AOB and AOA seem to greatly contribute to the process, while those present in NOB may play a minor role (Helbling et al., 2012; Su et al., 2021; Yu et al., 2018). Ammonia monooxygenase (AMO;

EC 1.14.99.39), present in AOB and AOA, is considered responsible for the biotransformation of multiple compounds (Fernandez-Fontaina et al., 2016; Men et al., 2017; Su et al., 2021; Wang et al., 2019; Yu et al., 2018), frequently through oxygen insertions resulting in hydroxylation reactions and sometimes in dehydrogenation or reductive dehalogenation (Helbling et al., 2012; Su et al., 2021). However, some studies have shown that the contribution to biotransformation of nitrifiers and AMO in activated sludge systems may often be over-estimated and belittle the role of other heterotrophic and nitrifying enzymes (Helbling et al., 2012; Men et al., 2017). In fact, the biotransformation of certain compounds in nitrifying activated sludge reactors could happen thanks to slow-growing heterotrophs (Achermann et al., 2018b; Gonzalez-Gil et al., 2021), suggesting that attention should also be paid to side sludge microbial activities. In this sense, high sludge retention times (SRT) are considered to improve OMPs biotransformation thanks to a broader microbial and functional diversity and a larger network of enzymatic activities produced by the additional presence of slow-growing microorganisms (Achermann et al., 2018b; Yuxin Wang et al., 2020). Achermann et al. (2018a) observed a characteristic trend between the SRT and the biotransformation of OMPs undergoing oxidation reactions, suggesting that those reactions may be catalyzed by enzymes less generally widespread among bacteria, that may become more abundant with the development of some groups of microorganisms. Differently, they observed a weak dependence between the SRT and the biotransformation of OMPs undergoing substitution reactions, indicating that enzymes broadly expressed by different microorganisms may be carrying them out, possibly because they are involved in mechanisms of central metabolism or general defense. Similarly, Torresi et al. (2018) did not find a positive correlation between some OMPs biotransformation and biodiversity, implying that the SRT and microbial richness may be only relevant when the biotransformation steps are performed by a limited number of taxa or enzymes.

The capability of heterotrophic cultures to biotransform OMPs is very relevant and it has been proven that just their basal expression may be sufficient to achieve high removal extents of multiple xenobiotics (Fischer and Majewsky, 2014). Their biotransformation potential comes from their ability to express a huge number of different enzymatic activities thanks to the wide range of carbon sources that they can assimilate, making it a difficult task to determine their abundance and importance (Kennes-Veiga et al., 2021b). Numerous biotransformation reactions documented in aerobic experiments

may have been carried out by heterotrophic enzymes, including addition, oxidation, substitution and cleavage reactions (Kolvenbach et al., 2014; Men et al., 2017). Besides, although nitrifying microorganisms frequently achieve higher biotransformation rates than heterotrophs (Wu et al., 2020), the latter display a superior metabolic versatility and a wider spectrum of reaction types that allow them to further biodegrade many OMPs and TPs (Khunjar et al., 2011; Wu et al., 2020). The most common enzymatic activities reported in aerobic conditions, along with further information, are described in Table 1.1.

Oxidoreductases (EC 1.-) are the most broadly reported enzymatic activities in OMPs biotransformation since oxidation reactions often represent the initial reaction step through the action of oxygenases, dehydrogenases, oxidases, reductases and peroxidases (Bilal et al., 2019; Nguyen et al., 2021; Stadlmair et al., 2018; Su et al., 2021). Non-specific mono- and dioxygenases are particularly relevant, having evidence of their participation in the oxidation of multiple compounds through reactions such as N- and S-oxidation, N- and O-dealkylation and hydroxylation (Achermann et al., 2018b, 2020; Gulde et al., 2016; Helbling et al., 2010a; Krah et al., 2016; Wu et al., 2020).

Several hydrolases (EC 3.-) are involved in OMPs biotransformation through the action of esterases (EC 3.1.-), glycosylases (EC 3.2.-), peptidases (EC 3.4.-) and amidases (EC 3.5.-) (Di Marcantonio et al., 2020; Helbling et al., 2010a; Krah et al., 2016; Nguyen et al., 2021; Stadlmair et al., 2018); and even activated-sludge protozoans have been reported to participate in the hydrolysis of some pharmaceuticals (Gulde et al., 2018). Esterases, including phosphatases, glycosidases and lipases, participate in the hydrolysis of many OMPs, such as in the cleavage of the lactone ring present in erythromycin, clarithromycin and azithromycin (Achermann et al., 2018b; Helbling et al., 2010a; Krah et al., 2016). Glycosylases of both intracellular and extracellular origin, including galactosidases (EC 3.2.1.23) and glucuronidases (EC 3.2.1.31), and peptidases (EC 3.4.-), such as cysteine peptidases (EC 3.4.22.-), serine peptidases (EC 3.4.21.-) and aspartic peptidases (EC 3.4.23.-), which have been reported to hydrolyze the amide bond of atenolol and bezafibrate, are also important for OMPs hydrolysis (Krah et al., 2016). Finally, amidases likely biotransform primary amides to carboxylic acids and hydrolyze secondary amides through amidohydrolases (EC 3.5.1.-); biotransform anilides through the action of aryl-acylamidases (EC 3.5.1.13); and participate in nitrile conversion to carboxylic acids through nitrilases (EC 3.5.5.-) (Achermann et al., 2018b; Helbling et al., 2010a; Vuono et al., 2016).

Transferases (EC 2.-) have been linked to OMPs biotransformation on many occasions. For instance, N-acyltransferases (EC 2.3.-) and glutathione-S-transferase (EC 2.5.1.18) can biotransform several amine and acetanilide-containing OMPs, respectively (Achermann et al., 2018b; Gulde et al., 2016). Besides, sulfonamide biotransformation is thought to occur through the pterin-sulfonamide conjugation pathway, likely initiated by dihydropteroate synthase (EC 2.5.1.15) and linked to cellular growth (Achermann et al., 2018b), and sulphotransferases (EC 2.8.2.-) and phosphotransferases (EC 2.7.-) may take part in diclofenac and macrolide biotransformation, respectively (Nguyen et al., 2021; Wu et al., 2020).

Other less commonly reported enzyme classes may also participate in OMPs biotransformation. Lyases (EC 4.-) may be responsible for the conversion of nitriles to amides through the action of nitrile hydratases (EC 4.2.1.84) and for the acetylation of some compounds after initial oxidation reactions thanks to carboxy-lyases (EC 4.1.1.) (Achermann et al., 2018b; Krah et al., 2016). Additionally, ligases (EC 6.-) could also play an important role, as observed for ibuprofen CoA ligase (EC 6.2.1.-) during ibuprofen removal in assays using a pure culture present in activated sludge (Vuono et al., 2016).

Table 1.1. Enzymes potentially involved in the aerobic cometabolic biotransformation of several OMPs.

OMPs	Reaction	Candidate enzymes	Methodology	References
Acetaminophen, Acetylsulfamethoxazole	Hydrolysis	Aryl-acylamidases (EC 3.5.1.13)	TPs	(Krah et al., 2016)
Acetanilides (alachlor, dimethenamid, flufenacet, metolachlor, propachlor)	Conjugation	Gluthatione-S-transferase (EC 2.5.1.18)	TPs	(Achermann et al., 2018b)
Acyclovir, penciclovir	Oxidation	Oxidoreductases (e.g., dehydrogenases, oxygenases, peroxidases) (EC 1.-)	TPs	(Prasse et al., 2011)
Anilides (N-(4-aminophenyl)-4-chlorobenzamide, N-phenyl-4-chlorobenzamide, N-(2-methylphenyl)acetamide)	Hydrolysis	Aryl-acylamidases (EC 3.5.1.13)	TPs	(Helbling et al., 2010a)
Primary, secondary amides (e.g., atenolol, rufinamide, bezafibrate, carbetamide)	Hydrolysis	Amidases (EC 3.5.1-)	TPs	(Helbling et al., 2010b) (Achermann et al., 2018b)
Secondary, tertiary amides (e.g., tebutam, fenhexamid, valsartan, diazepam)	N-dealkylation	Oxidoreductases (e.g., Monooxygenases (EC 1.13.- // EC 1.14.-), hydrogenases (EC 1.1.- // EC 1.2.-)	TPs	(Helbling et al., 2010a) (Helbling et al., 2010b) (Achermann et al., 2018b)
Primary and secondary amines (e.g., feniramine, primaquine, fluoxetine)	N-dealkylation/ Hydroxylation	Monooxygenases (e.g., Cytochrome P450; Flavin containing-monooxygenases) (EC 1.13.- // EC 1.14.-)	TPs	(Gulde et al., 2016)
	N-acylation (e.g., N-acetylation, N-formylation, N-succinylation)	N-acyltransferase (EC 2.3.1.-)	TPs	(Gulde et al., 2016)
Tertiary amines (e.g., venlafaxine, pargyline, deprenyl)	N-oxidation/ hydroxylation/ N-dealkylation	Monooxygenases (e.g., Cytochrome P450; Flavin monooxygenases) (EC 1.13.- // EC 1.14.-)	TPs	(Gulde et al., 2016)

Atenolol, bezafibrate	Hydrolysis	Endopeptidases (EC 3.4.-)	Inhibition assays	(Krah et al., 2016)
Azoxystrobin Isoproturon Chlortoluron	Hydrolysis	Protozoan hydrolases (EC 3.-)	TPs	(Gulde et al., 2018)
Bisphenol-A	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Omics – Real-time PCR	(Cydzik-Kwiatkowska et al., 2020)
10-hydroxy-carbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, Oxcarbazepine	Oxidation	Oxidoreductases (EC 1.-)	TPs	(Kaiser et al., 2014)
Codeine	Oxidation	Oxidoreductases (EC 1.-)	TPs	(Wick et al., 2011)
EEnB (N,N-diethyl-4-nitrobenzamide)	Nitro reduction	Nitroreductase (EC 1.5.1.34)	TPs	(Helbling et al., 2010a)
Erythromycin	Hydrolysis	Esterases (EC 3.1.-)	In vitro assays + TPs	(Krah et al., 2016)
Isoproturon, ranitidine, venlafaxine	S-oxidation, N-oxidation, N-dealkylation	Archaeal ammonia monooxygenase (EC 1.14.99.39)	Enzymatic inhibition + TPs + Omics	(Helbling et al., 2012)
Nitriles (bromoxynil, acetamiprid)	Nitrile hydration	Nitrile hydratase (EC 4.2.1.84)	TPs	(Achermann et al., 2018b)
Oseltamivir	Hydrolysis	Esterases (EC 3.1.1.-)	TPs	(Helbling et al., 2010b) (Helbling et al., 2010a)
Propachlor	Conjugation	Glutathione-S-transferase (EC 2.5.1.18)	TPs	(Helbling et al., 2010a) (Helbling et al., 2010b)
Sulfonamides (sulfamethoxazole, sulfadiazine, sulfapyridine, sulfathiazole, sulfamethazine)	Conjugation	Dihydropteroate synthase (EC 2.5.1.15)	TPs	(Achermann et al., 2018a)
Thioethers (irgarol, terbutryn, ranitidine)	Oxidation	Monooxygenase (EC 1.13.- // EC 1.14.-)	TPs	(Achermann et al., 2018b)
Trimethoprim	Demethylation	Monooxygenases (EC 1.13.- // EC 1.14.-)	TPs	(Krah et al., 2016)

Trinexapac-ethyl	Hydrolysis	Hydrolases (e.g., esterases) (EC 3.-)	TPs	(Achermann et al., 2018b)
Celestolide, galaxolide, estrone, estradiol, Diclofenac, Nonylphenol	Oxidation	Oxidoreductases (EC 1.-)	TPs	(Kennes-Veiga et al., 2021b)
Celestolide, nonylphenol	Dehydrogenation	Alcohol dehydrogenases/Oxidases (EC 1.1.-, EC 1.13.-, EC 1.14.-)	TPs	(Kennes-Veiga et al., 2021b)
Diazepam	Demethylation	Oxidoreductases (EC 1.-)	TPs	(Kennes-Veiga et al., 2021b)
17α-ethinylestradiol (EE2)	Oxidation	Mono- and dioxygenases (EC 1.13.- // EC 1.14.-)	Enzymatic inhibition + TPs	(Khunjar et al., 2011)
Fluoxetine	Oxidation	Oxidoreductases (EC 1.-)	TPs	(Kennes-Veiga et al., 2021b)
	Demethylation	Oxidoreductases (EC 1.-)	TPs	(Kennes-Veiga et al., 2021b)
	Deamination	Amine oxidase (EC 1.4.-)	TPs	(Kennes-Veiga et al., 2021b)
	Conjugation	N-acyltransferases (EC 2.3.-)	TPs	(Kennes-Veiga et al., 2021b)
Ibuprofen	Hydroxylation/ Carboxylation/ Decarboxylation	Oxidoreductases (EC 1.-)/ Lyases (EC 4.-)/ Ligases (EC 6.-)	Enzymatic inhibition + TPs	(Jia et al., 2020)
Sulfamethoxazole	Conjugation	Dihydropteroate synthase (EC 2.5.1.15)	TPs	(Kennes-Veiga et al., 2021b) (Achermann et al., 2018a)
Trimethoprim	Oxidation	Mono- and dioxygenases (EC 1.13.- // EC 1.14.-)	Enzymatic inhibition + TPs	(Khunjar et al., 2011)
	Hydrolysis	Aminopyrimidine aminohydrolase (EC 3.5.99.-)	TPs	(Kennes-Veiga et al., 2021b)
Bisphenol A, iohexol, irgarol, naproxen, terbutryn	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Enzymatic inhibition	(Margot et al., 2016)
Cephalexin	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Omics – Quantitative PCR	(Wang et al., 2019)

EE2	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Enzymatic inhibition + TPs	(Khunjar et al., 2011)
Ibuprofen	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Enzymatic inhibition + TPs	(Fernandez-Fontaina et al., 2016)
Phenylureas, thioethers, amidines (e.g., asulam, monuron, acetamiprid)	Oxidation	Ammonia monooxygenase (EC 1.14.99.39)	Enzymatic inhibition	(Men et al., 2017)
Sulfonamides (sulfadiazine, sulfamethazine, sulfamethoxazole)	Deamination	Deaminases (EC 3.5.-)	TPs	(L. J. Zhou et al., 2019)
	Hydroxylation/ Nitration	Ammonia monooxygenase (EC 1.14.99.39)		

1.4. APPROACHES TO DETERMINE KEY ENZYMES

A huge amount of enzymes participate in biological wastewater treatments and, thanks to functional redundancy, the same metabolic process can be conducted by several microorganisms and enzymatic routes (Sambamoorthy and Raman, 2018). Therefore, linking the biotransformation of OMPs with specific enzymatic activities is a complex task that entails a deep understanding of the biological processes and the development of appropriate experimental strategies. In this section, the main approaches employed to identify the key enzymes are described and analyzed from a critical point of view (Fig. 1.2).

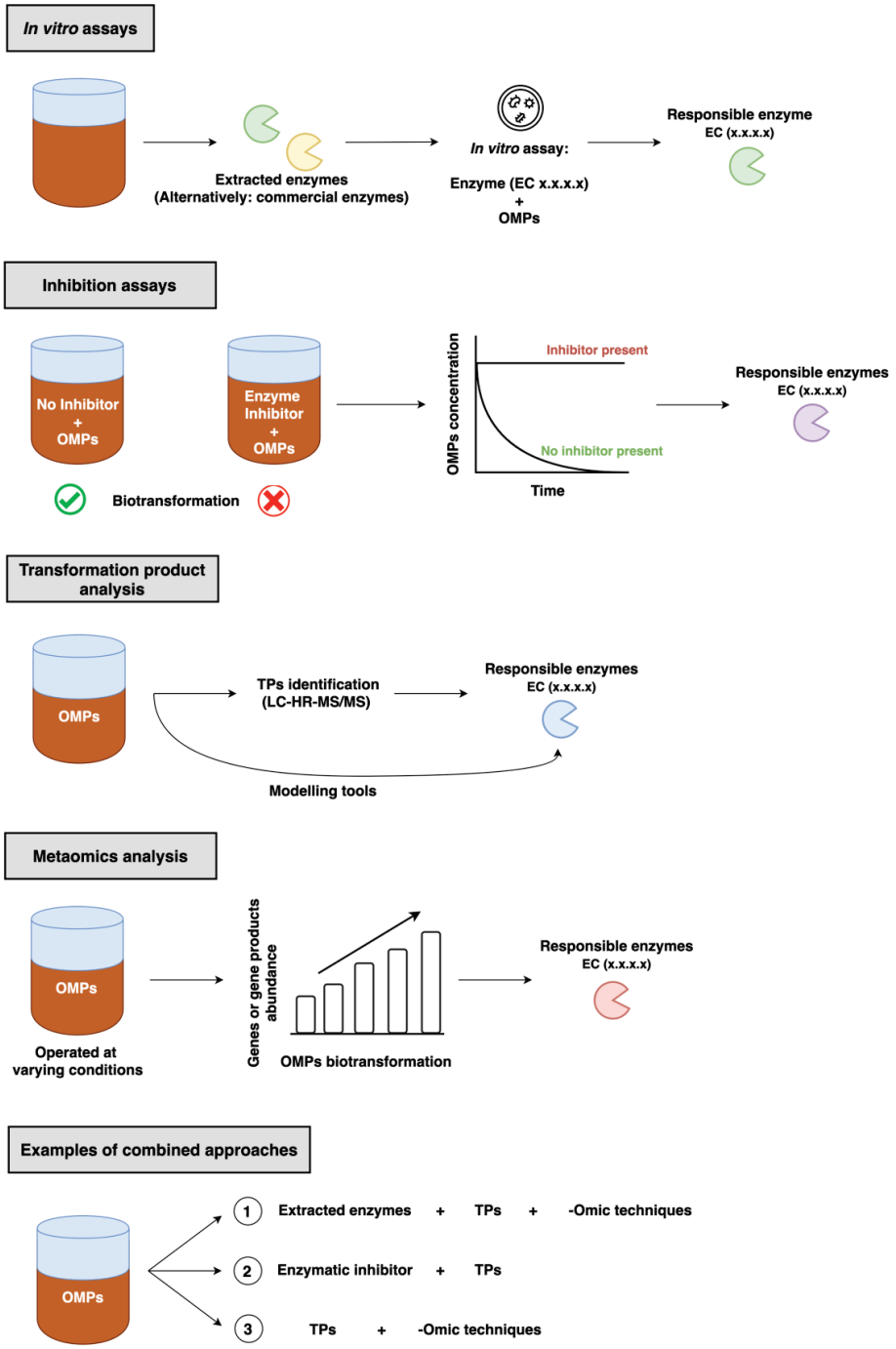

Fig. 1.2. Frequent approaches applied to determine the enzymatic activities involved in OMPs biotransformation.

1.4.1. *In vitro* assays

In vitro assays refer to a methodology where cell-free enzymes are cultured in a controlled and artificial environment outside of the living microorganism to address their role on OMPs biotransformation. So far, two approaches of this technique have been followed: *(i)* use of a sole purified enzyme, either obtained commercially (Gonzalez-Gil et al., 2017) or in the laboratory from a specific microorganism (Prior et al., 2010; Xu et al., 2015); and *(ii)* use of an enzymatic lysate directly extracted from the reactor biomass and composed of a mixture of enzymes (Gonzalez-Gil et al., 2019a; Krah et al., 2016; Zumstein and Helbling, 2019). The procedure for both strategies is summarized in Fig. 1.3. When using sole enzymes, significant efforts are needed to select the suitable ones. Firstly, the enzyme should be representative of the biological system under study and show a relevant activity in the bioreactor (Gonzalez-Gil et al., 2017), which requires a deep understanding of the metabolic pathways and species involved in the process. Secondly, it has to be feasible to obtain the enzyme in a purified form. In the case of using a cell-free lysate with a cocktail of enzymes directly extracted from the biomass reactor, different procedures can be conducted. If the goal is to study extracellular enzymes, cell lysis is not needed (Zumstein and Helbling, 2019), but it should be performed to extract intracellular enzymes (Krah et al., 2016). To recover membrane enzymes and disassemble enzymes from the extracellular polymeric substance, surfactants and cation exchange resins should be added, respectively. Once the cell-free lysate is obtained, the supply of cofactors and inhibitors should be considered to promote and suppress specific enzymatic activities, which could aid in the identification of specific enzymatic activities related to OMPs removal (Gonzalez-Gil et al., 2019a).

The use of sole purified enzymes in *in vitro* assays allows to test and prove specific biotransformation pathways without the interference of other enzymes, which could happen when using an enzymatic lysate, allowing to narrow down the prediction and identification of TPs. Successful results with commercially purified enzymes were reported by Gonzalez-Gil et al. (2019b, 2017), who demonstrated that acetate kinase and hexokinase could transform OMPs with carboxyl and hydroxyl groups and moderate steric hindrance. According to Bisswanger (2011), thousands of enzymes exist, but less than 15 % are described in detail and only hundreds are commercially available. Even if so, enzymatic substrate selectivity and affinity can differ depending on the microorganism synthesizing the enzyme. For instance, acetate kinase from E. coli is more

substrate-specific than that from M. thermophila (Bock et al., 1999), which might result in a lower affinity for certain cosubstrates (i.e., OMPs) and explain why a reduced BPA biotransformation was achieved when using the former instead of the latter, which is present in anaerobic digesters (Gonzalez-Gil et al., 2019b, 2017).

An alternative to commercially purified enzymes is to isolate microbial species from a bioreactor, identify the enzymes of interest and purify them to perform *in vitro* experiments. There are some studies where cytochrome P450 enzymes of specific bacteria were expressed in E. coli to perform *in vitro* experiments that demonstrated their ability to hydroxylate diclofenac (Prior et al., 2010; Xu et al., 2015); although the microorganisms studied do not belong to wastewater biological systems. This methodology enables testing a wider spectrum of enzymes, but its experimental success depends on complex challenges ranging from isolating and cultivating pure microorganisms to expressing and purifying the enzymes. In fact, the statement of Fischer and Majewsky (2014a) pointing out that the isolation of target enzymes present in WWTPs has not been documented, is still valid nowadays. The closest approach reported is the obtainment of a cocktail of extracted enzymes from biomass of activated sludge systems (Krah et al., 2016; Zumstein and Helbling, 2019) and anaerobic digesters (Gonzalez-Gil et al., 2019b), which allowed working with a wide variety of native enzymes. This method, despite being a simplification of *in vivo* processes, seems a more realistic strategy than working with individual enzymes. Yet, it might be difficult to ascertain which are the specific enzymes responsible for OMPs biotransformation. In fact, although the mentioned studies provided sound arguments about the candidate enzymes responsible for the observed biotransformation reactions, their identity could not be irrefutably proved. Moreover, another limitation of this procedure is that it is not possible to extract all the enzymes nor perform long-term experiments since the enzymes can rapidly lose their activity, which could compromise the comparison with results *in vivo*. Furthermore, *in vitro* assays are limited in properly representing complex biological processes, and it cannot be fully assured that reactions will occur in the same way and extent as under real bioreactor conditions, where biomass and substrate heterogeneity is huge and competition and inhibition mechanisms might happen among enzymes and microorganisms. Nonetheless, their results are undoubtedly valuable to solve the puzzle of OMPs biotransformation.

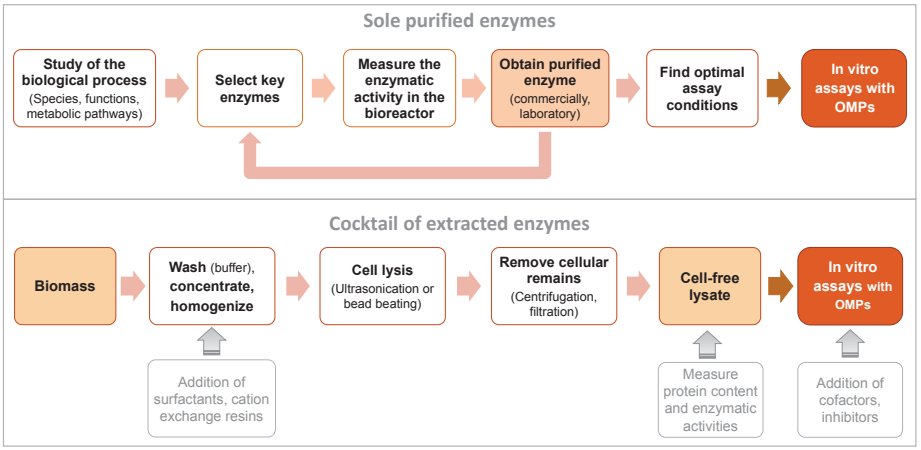

Fig. 1.3. Main stages needed to perform *in vitro* assays with sole purified enzymes or a cell-free lysate.

1.4.2. Use of inhibitors in microbial and enzymatic cultures

The use of enzymatic inhibitors is a fast and simple approach to determine the relative contribution of specific enzymatic activities present in a microbial population. The methodology consists in linking a decrease in OMPs biotransformation to the activity of the inhibited enzyme, which offers an interesting starting point to unravel OMPs biotransformation pathways. Results can imply that the inhibited enzymes and microorganisms are responsible for the biotransformation (direct involvement) or that their inhibition leads to the subsequent suppression of the microbial and enzymatic activities carrying out the process (indirect involvement) (Men et al., 2017). This approach is particularly useful when the inhibitor is very enzyme-specific, making its selection a key aspect that requires knowledge on the inhibited enzymes and the associated biotransformation reactions. In fact, a common problem occurs when the enzymatic specificity of the inhibitor is broader than expected, possibly because it had not been tested before with as many enzymes as those present in wastewater mixed microbial cultures (Gonzalez-Gil et al., 2019b; Helbling et al., 2012). Thus, performing multiple experiments with an extended range of inhibitors that theoretically hinder the same reaction could help to increase confidence in the results (Men et al., 2017).

The role of AOB in OMPs biotransformation has typically been determined through inhibition studies with allylthiourea (ATU) (Fernandez-Fontaina et al., 2016; Margot et al., 2016; Wu et al., 2020). However, Men et al. (2017), who used two ammonia oxidation

inhibitors, ATU and octyne (OCT), concluded that ATU could overestimate the role of AOB by not being its inhibitory effect as specific as previously thought. They observed that 17 compounds showed significantly reduced biotransformation with ATU compared to OCT, particularly those containing thioether and phenylurea groups. Since they determined that ATU barely affected heterotrophic respiration and the essential heterotrophic enzymes implicated in energy conservation and central metabolism, they suggest that ATU may have inhibited other non-essential heterotrophic enzymes possibly involved in OMPs biotransformation.

Inhibition studies have also been performed to elucidate the role of other enzymes and microorganisms. Krah et al. (2016) carried out experiments with extracted native enzymes from activated sludge and, using peptidase inhibitors, determined the involvement of peptidases in the hydrolysis of amide bonds present in OMPs. They used three inhibitors targeting the action of endopeptidases (E-64 for cysteine peptidases (EC 3.4.22.-); 4-benzenesulfonyl fluoride hydrochloride (AEBSF) for serine peptidases (EC 3.4.21.-) and pepstatin A for aspartic peptidases (EC 3.4.23.-)) and documented their involvement in the biotransformation of atenolol and bezafibrate; although they point out the need of performing further studies for confirmation since the inhibitors could have affected the activity of other non-endopeptidase enzymes. Gonzalez-Gil et al. (2019b) performed inhibition assays *in vitro* with extracted native enzymes from anaerobic sludge using E-64, AEBSF and pepstatin A as peptidase inhibitors and castanospermine and 2-bromoethanesulfonate (BES) as glycosidase and methanogenic inhibitors, respectively. They observed that the biotransformation of clarithromycin, climbazole, citalopram and erythromycin was impaired under the action of the peptidase inhibitors and that atenolol biotransformation was fully inhibited under the action of AEBSF, suggesting the participation of peptidase enzymes in their removal. However, they also reported that the biotransformation of acetyl-sulfamethoxazole was considerably inhibited under the action of BES even though methyl-CoM reductase is unlikely involved in the process, indicating that the action of the inhibitor is more unspecific than expected. Moreover, reduced removal of erythromycin and clarithromycin in assays with castanospermine was not observed despite being good candidates for glycosylase action due to their cladinose moiety, suggesting that other glycosylases not affected by the inhibitor could participate in the biotransformation (Gonzalez-Gil et al., 2019b).

1.4.3. Transformation product analysis

As previously mentioned, biotransformation of most OMPs in WWTPs does not lead to complete mineralization, giving rise to the formation of TPs. Since OMPs biotransformation is mainly influenced by the affinity of their chemical structure with the unspecific enzymes expressed during primary substrate biodegradation (Han Tran et al., 2017), TPs identification and prediction would be possible if the enzymatic cycles were deeply understood and the structures of growth substrates and OMPs could be correlated. In like manner, TPs structure analysis and elucidation can be an excellent tool to obtain information on OMPs biodegradability and link enzymatic activities taking part during biotransformation (Table 1.1). However, until recently, scarce information was available due to the challenging task of identifying and quantifying unknown TPs present at extremely low concentrations in complex matrices (L. Zhang et al., 2013). Fortunately, the development and optimization of versatile analytical tools have helped to search and identify many suspect and non-target compounds with reasonable accuracy and certainty (Fenner et al., 2021; Gulde et al., 2016).

Determining key enzymes through TPs analysis provides several advantages, such as: the experimental design is generally simple; lab results can realistically be extrapolated to WWTPs (Kern et al., 2010); the risks of finding and acquiring commercial enzymes for *in vitro* assays or of suffering enzymatic activity loss when working with enzymatic lysates are avoided (section 1.4.1.); and the substantial costs of other techniques are eluded (section 1.4.4.). For such reason, to date, most candidate enzymes involved in OMPs biotransformation have been suggested applying this methodology (Achermann et al., 2018b; Gulde et al., 2016; Kennes-Veiga et al., 2021b; Yu et al., 2018). However, TPs elucidation cannot lead to a direct and complete confirmation of the responsible enzymatic activities and certain constraints might affect TPs detection and identification through mass spectrometry approaches, such as when: they are present at concentrations below the detection limit; they possess molecular masses outside the mass range of the full scan; they are highly unstable and readily transformable; their structures are too simple and lead to analytical errors; they have compound-specific properties limiting the analytical ionization efficiency; they have structural isomers; interfering ions with the same mass as the TPs are present; and there is a lack of reference standards that allow achieving higher confidence levels in the proposed TPs structures (Helbling et al., 2010b; Kern et al., 2010; Schymanski et al., 2014). Thus, it is advisable to combine results

from several approaches, such as *in vitro* assays, enzymatic inhibition and TPs analysis, which allows determining key enzymes with much higher confidence, as performed to identify the contribution of serine proteases (EC 3.4.21.-) during the biotransformation of atenolol in anaerobic conditions (Gonzalez-Gil et al., 2019b).

Filling the knowledge gap of TPs formation and linking them to responsible enzymes just with laboratory studies is a considerable challenge since such assays can be expensive and time-consuming, biotransformation is influenced by multiple process and environmental factors and microorganisms may develop new catabolic pathways over time (Kolvenbach et al., 2014; Men et al., 2017; Yuxin Wang et al., 2020). Therefore, the use of modeling tools, such as PathPred (Moriya et al., 2010), CRAFT (CRAFT, 2009), OECD Toolbox (OECD, 2020), enviPath (Wicker et al., 2016) and the EAWAG-PPS (EAWAG-BBD/PPS), which are based on biotransformation rules extracted from microbial metabolic pathways and enzymatic reactions reported in the literature, may provide an appropriate and cost-effective approach to help predict transformation reactions at the different molecular functional groups (Helbling et al., 2010b). They can contribute to building pathway knowledge, determine the biodegradability of OMPs and TPs, and perform risk assessments. Besides, they are extremely useful when creating lists of expected TPs in suspect-screening approaches (Achermann et al., 2018b; Gulde et al., 2016; Kennes-Veiga et al., 2021b). However, their main constraint is that they are exceptionally sensitive but poorly selective, leading to the prediction of too many TPs and causing numerous false positives, likely due to the limited data available of microbial processes and for not considering the effects caused by moieties surrounding the target functional groups in the biotransformation rules. Another limitation is that most rules and microbial pathways are based on literature information obtained from studies performed with pure or enriched cultures, unspecific environmental conditions or where xenobiotics are used as primary substrates, resulting in biotransformation reactions unlikely to happen during the cometabolic biotransformation of trace-level OMPs by mixed cultures (Gulde et al., 2016). Thus, combining modeling tools and lab experiments under defined and environmentally realistic conditions is necessary to improve the prediction of TPs and learn about new biotransformation pathways.

1.4.4. Omics approach

The recent evolution and improvement of sequencing techniques, the increased knowledge on sequence information and the development of protein and genome

databases, such as Eawag-BBD/PPS and enviPath (EAWAG-BBD/PPS; Wicker et al., 2016), have allowed using meta-omics association studies to elucidate OMPs biotransformation pathways and set up hypotheses of potentially involved enzymatic activities (Table 1.1) (Krah et al., 2016). With that purpose, metagenomics, reporting which microorganisms are present in a sample and its metabolic potential; metatranscriptomics, offering precise information on the microbial functions happening at a given time; and proteomics analyses, showing the active enzyme pool present in the microbiome, have been increasingly performed. Metagenomics techniques have been widely applied in experiments with OMPs through modern high-throughput DNA sequencing technologies, while the use of metatranscriptomics and metaproteomics is more recent and there is debate about their suitability (Fenner et al., 2021). Metatranscriptomics has a higher sensitivity to detect low-abundance gene transcripts than metaproteomics to detect expressed proteins, and transcripts have been shown to correlate properly with the protein abundance level, but it generally implies a higher cost. Additionally, metaproteomics may provide a higher potential to obtain mechanistic insights thanks to describing the enzyme pool more directly (Achermann et al., 2020; Fenner et al., 2021), but protein identification requires metagenomic data from the samples, along with detailed interpretation and annotation (Fenner et al., 2021).

The introduction and accumulation of OMPs in bioreactors affect microbial community structure and leads to changes in the expression levels of biodegradation genes and gene products, as observed by Harb et al. (2016) using high-throughput 16S rRNA gene sequencing, metatranscriptomics and gene databases. Thus, -omics association studies have been broadly used to find biomarkers that can help to understand and predict the capacities of microbial communities and the influence of specific WWTPs parameters (Helbling et al., 2012; David R. Johnson et al., 2015a). For example, Cydzil-Kwiatkowska et al. (2020) determined a linear correlation between BPA concentration and the expression levels of the gen *bisdA*, which encodes for ferredoxin. Similarly, Zhou et al. (2015), in an experiment with *Sphingobium sp. BiD32* at varying concentrations of BPA, studied its pathway and key enzymes through genomic, proteomic and TPs analysis. They observed the upregulation of 43 proteins belonging to the dehydrogenase, dioxygenase, hydratase, hydroxylase and cycloisomerase class; confirmed that p-hydroxybenzoate hydroxylase was involved in the first biotransformation step and identified the respective TP as a genetic biomarker for BPA biotransformation. Helbling

et al. (2015), with a multivariate model and bacterial 16S rRNA analysis, determined that specific phylogenetic groups can also serve as biomarkers of microbial community activity towards OMPs biotransformation, although taxonomic biomarkers may only be useful when there is no functional redundancy and biotransformation is not extensively distributed among taxa. In this sense, Vuono et al. (2016), analyzing 16S rRNA genes and rRNA gene expression, observed that rare taxa had higher ratios of rRNA to rDNA and a superior protein synthesis potential, which suggests their relevant role in reactor performance and OMPs biotransformation, and confirms that functional and taxonomic richness can positively influence OMPs removal (Stadler et al., 2018).

The main advantage of meta-omics techniques is that they intend to characterize the complete set of genes or gene products present in a microbial community at a given time, avoiding the need for the specific and robust knowledge required for the previously described approaches (sections 1.4.1, 1.4.2 and 1.4.3) (Achermann et al., 2020; Krah et al., 2016). Besides, meta-omics association not only informs about key enzymatic activities but also points towards specific microorganisms responsible for biotransformation and provides information on the subcellular location and organismal origin of candidate enzymes (Krah et al., 2016). Such knowledge is highly important since it can allow predicting changes in OMPs biotransformation rates and pathways (Stadler et al., 2018) given that: *(i)* biotransformation steps may be carried out by different organisms (Achermann et al., 2020; Helbling et al., 2015; David R. Johnson et al., 2015a); *(ii)* enzymes of different microbial origins can possess varying catalytic activities (Gonzalez-Gil et al., 2019b, 2017; David R. Johnson et al., 2015a); *(iii)* the same TP of an OMP can be produced by different microorganisms and enzymes (Zhou et al., 2015); and *(iv)* the same gene may associate with different enzymatic activities depending on the reactor configuration (Harb et al., 2016).

The essential limitation of -omic association mining is the generation of a large number of false positives that lead to non-causal correlations, adding great complexity to the identification of genes or gene products of interest if hypotheses are not reduced when the large datasets are obtained (Achermann et al., 2020; David R. Johnson et al., 2015b). Moreover, a direct statement on the involvement of an enzyme in OMPs biotransformation is often not possible since, for instance, the upregulation of transcripts does not always imply an increase in enzymatic activity and the physiological functions of promiscuous enzymes cannot be consistently predicted based on the knowledge of the

genes encoding them (Fenner et al., 2021; Kolvenbach et al., 2014; Stadlmair et al., 2018). Furthermore, statistical power is often lacking due to the huge amount of data gathered from a low number of samples; -omics analyses are still not broadly accessible and its costs usually do not allow their application to large sets of samples; and the adequacy of this methodology for enzymatic elucidation when OMPs are present at trace levels and lead to a lack of metabolic pathway induction is under debate (Fenner et al., 2021). Thus, results from experiments using extracted native enzymes could be particularly useful to complement and confirm the information obtained through -omic techniques, supporting the links made between gene products and OMPs biotransformation (Krah et al., 2016).

Overall, all technologies described in section 1.4 have many advantages and disadvantages, suggesting that the best way to limit the number of hypotheses, to establish causality and to strengthen the confidence in the identification of the microorganisms and enzymes participating in OMPs biotransformation is a synergistic application of all approaches, as positively observed in recent studies (Achermann et al., 2018b, 2020; Zhao et al., 2020).

1.5. OBJECTIVES AND STRUCTURE

Based on the research needs set out in the introduction, this section presents the objectives of the thesis and describes its structure.

1.5.1. Main objective

The main objective of this PhD dissertation is to improve the understanding of the cometabolic biotransformation mechanisms that OMPs undergo under aerobic heterotrophic conditions in WWTPs. This knowledge will eventually help to optimize process conditions in wastewater treatment facilities to maximize their removal.

1.5.2. Research questions

To fulfill the main objective of the thesis and fill in the gaps of the existing knowledge on the cometabolic biotransformation of OMPs under aerobic heterotrophic conditions, several research questions were devised:

- What is the potential of aerobic heterotrophs to biotransform OMPs showing a wide range of physico-chemical properties?

- Is cometabolism the main responsible mechanism for OMPs biotransformation under aerobic heterotrophic conditions?
- How does the heterotrophic activity affect the biotransformation efficiency and rate of OMPs?
- What are the main OMPs biotransformation reactions carried out by heterotrophic microorganisms? What are the key enzymatic activities involved in the cometabolic biotransformation of OMPs under aerobic heterotrophic conditions?
- Is there a specific heterotrophic microbial population playing a key role during OMPs biotransformation?

1.5.3. Research objectives

After a solid state-of-the-art analysis and review on the cometabolic biotransformation of OMPs in WWTPs, the previously stated research questions were translated into the following research objectives:

- To determine the capabilities of aerobic heterotrophs to biotransform OMPs of multiple physico-chemical properties.
- To analyze the relevance of cometabolism during the biotransformation of OMPs under aerobic heterotrophic conditions.
- To assess the influence of the primary substrate consumption (heterotrophic activity) on the OMPs biotransformation efficiency and kinetics.
- To discern OMPs biotransformation pathways under aerobic heterotrophic conditions through the identification of TPs.
- To identify the key microorganisms and enzymes involved in the aerobic heterotrophic biotransformation of OMPs.

1.5.4. Thesis structure

The structure of the thesis aims at deepening step by step into the underlying OMPs cometabolic biotransformation mechanisms of aerobic heterotrophic microorganisms.

Firstly, in **Chapter 3**, the capabilities of the aerobic heterotrophs to biotransform OMPs are studied, assessing whether cometabolism is the main responsible mechanism behind the biotransformation process. Additionally, the influence of the heterotrophic activity on the biotransformation kinetics and, specifically, on the biotransformation kinetic constant of the OMPs is analyzed.

Chapter 4 aims at comparing the capacities of aerobic heterotrophic microorganisms to remove OMPs with respect to purely nitrifying environments and activated sludge systems combining both nitrifying and heterotrophic activities. Besides, the main OMPs biotransformation reactions carried out by heterotrophic microorganisms are identified and linked to the enzymatic activities likely responsible to catalyze such reactions.

In **Chapter 5**, an in-depth analysis of sulfamethoxazole biotransformation under aerobic heterotrophic conditions is performed. For that purpose, genomic, metaproteomic and TPs analyses were combined aiming at identifying the bacterial populations and enzymatic activities responsible for such biotransformation.

Finally, **Chapter 6** jointly analyses the results obtained in previous chapters and points out the main novelties and advances that the present thesis has achieved regarding OMPs biotransformation. Furthermore, current research gaps and opportunities are identified and suggestions for future research are provided.

CHAPTER 2. MATERIALS, METHODS AND EQUIPMENT

SUMMARY

In this chapter, the feedstock, the experimental set-ups, the analytical methods and the main calculations applied to characterize the operation of the bioreactors used in this PhD thesis are presented and described.

2.1. FEEDSTOCK PREPARATION

2.1.1. Organic micropollutants

A total of 20 environmentally relevant compounds commonly present in wastewater and covering a wide variety of functional groups, physicochemical properties and applications were used in this work (Table 2.1). The compounds were: the antibiotics erythromycin (ERY), roxithromycin (ROX), trimethoprim (TMP), sulfamethoxazole (SMX); the anti-inflammatories ibuprofen (IBP), naproxen (NPX), diclofenac (DCF); the neurodrugs carbamazepine (CBZ), diazepam (DZP), fluoxetine (FLX); the biocide triclosan (TCS); the musk fragrances celestolide (ADBI), galaxolide (HHCB), tonalide (AHTN); the endocrine disruptors estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and the xenoestrogens bisphenol A (BPA), 4-octylphenol (OP) and 4-nonylphenol (NP). The OMPs were purchased from Sigma-Aldrich (Germany), except for the musk fragrances, which were acquired from Ventos (Spain). Depending on the substance, stock solutions were prepared in HPLC-grade acetone or methanol and stored at -20°C.

Table 2.1. Physicochemical properties (obtained from DrugBank, PhysProp and Pubchem databases), application and wastewater occurrence of the selected OMPs (Besha et al., 2017; Clara et al., 2011; Luo et al., 2014; Petrie et al., 2014; Tran et al., 2018; Verlicchi et al., 2012; Verlicchi and Zambello, 2015).

OMPs	WWTPs occurrence ($\mu g\ L^{-1}$)	MW ($g\ mol^{-1}$)	s ($mg\ L^{-1}$)	H ($Pa\ m^3\ mol^{-1}$)	pKa	log K_{ow}	log K_d
E1	0.002 – 0.7	270.4	30	$3.8 \cdot 10^{-5}$	10.3	3.1	2-2.8
E2	0.01-3	272.4	3.6	$3.6 \cdot 10^{-5}$	10.3	4	2.6-3.0
EE2	0.002-0.07	296.4	11.3	$7.9 \cdot 10^{-7}$	10.3	3.7	2.3-3.2
ADBI	LOQ-0.1	244.4	0.2	$1.8 \cdot 10^{3}$	-	5.7	3.3-3.7
HHCB	0-13	258.4	1.8	1.1	-	5.9	3.2-3.7
AHTN	0-5.5	258.4	1.3	1.4	-	5.7	3.4-3.8
TCS	0.4-4.2	289.5	10	$2.1 \cdot 10^{-3}$	7.9	4.8	3.3-5
IBP	0.8-373	206.3	21	$1.5 \cdot 10^{-2}$	5.3	4	0.2-2.4
ROX	0.01-0.21	837.1	0.02	$5.0 \cdot 10^{-26}$	12.5	2.8	1.7-2.7
ERY	0.06-10	733.9	2	$5.4 \cdot 10^{-24}$	8.9	3.1	1.5-1.9
NPX	0.04-52	230.3	15.9	$3.4 \cdot 10^{-5}$	4.2	3.2	1.0-2
SMX	LOQ-7.9	253.3	1	$6.4 \cdot 10^{-8}$	6.2	0.9	0.2-2
TMP	LOQ-4.7	290.3	1	$2.4 \cdot 10^{-9}$	7.1	0.9	1.4-2.5
CBZ	LOQ-21	236.3	17.7	$1.1 \cdot 10^{-2}$	15.9	2.5	0.1-1.7
DZP	LOQ-21	284.7	50	$3.6 \cdot 10^{-3}$	3.4	2.8	1.3-2.4

2.1.2. Synthetic feeding mixture

All the experiments described in this thesis were performed using a synthetic feeding mixture, which was based on sodium acetate and acetic acid as primary carbon source (in concentrations that ensured operation at neutral pH), ammonium chloride, potassium dihydrogen phosphate, calcium chloride and magnesium sulfate (Table 2.2). Acetate was selected as the carbon source for being an easily biodegradable substrate, optimal for microbial growth and directly metabolized through the Krebs cycle (Nelson and Cox, 2017). Even though distinct carbon sources lead to the expression of different enzymatic activities, the mineralization of organic matter typically requires the involvement of the Krebs cycle. Thus, the use of acetate ensures that the enzymatic activities present in the

bioreactor are also present in WWTPs that deal with more complex carbon substrates. Moreover, other trace nutrients were also added to promote the growth of aerobic heterotrophic microorganisms (Table 2.3) and allylthiourea solution was added in the feeding with a concentration of 5 mg L^{-1} to avoid nitrification.

Table 0.2. Feeding composition and concentrations used to obtain a chemical oxygen demand (COD) of 0.2 g L^{-1}. In the experiments with other COD concentrations, the concentrations were calculated proportionally.

Compounds	mg L^{-1}
Sodium acetate ($CH_3COONa \cdot 3H_2O$)	210
Acetic Acid (CH_3COOH)	95
Ammonium chloride (NH_4Cl)	40
Potassium dihydrogen phosphate (KH_2PO_4)	10
Calcium chloride ($CaCl_2$)	5.5
Magnesium sulfate ($MgSO_4$)	5.5

Table 0.3. Trace metals composition and concentrations used in the feeding of the aerobic heterotrophic reactor.

Compounds	mg L^{-1}
$FeCl_3 \cdot 6H_2O$	0.15
H_3BO_3	0.015
$CuSO_4 \cdot 5H_2O$	0.003
KI	0.003
$ZnSO_4 \cdot 7H_2O$	0.012
$CoCl_2 \cdot 6H_2O$	0.015
$MnCl_2 \cdot 4H_2O$	0.012

2.2. EXPERIMENTAL DESIGN

Different types of reactor configurations (continuous, sequential batch and batch reactors) were operated to understand the cometabolic biotransformation of a range of OMPs under aerobic heterotrophic conditions.

2.2.1. Continuous reactors

5 L continuously stirred lab-scale reactors connected to a 2 L settler were used in **Chapters 3 and 4**, while a reactor of 2 L connected to a 1 L settler was used in **Chapter 5** (Fig. 2.1.). The reactors were inoculated with sludge from a conventional activated sludge reactor of a WWTP near Santiago de Compostela (Spain), setting an in-reactor biomass concentration of approximately 1.0 g volatile suspended solids (VSS) L^{-1}, and operated at 25ºC. The WWTP is designed for 184000 population equivalents, receives an influent COD ranging between 0.2 - 0.7 g L^{-1} and operates with approximate SRT and hydraulic retention time (HRT) values of 10 d and 8 h, respectively. In the reactors, the HRT was set to 1 d to provide sufficient time to the microorganisms to achieve the maximum biotransformation they are capable of, at the same time as minimizing possible changes in the microbial population caused by an HRT too long. Besides, the SRT was maintained around 5 d to minimize the presence of slow-growing microorganisms and favor the heterotrophic activity (Achermann et al., 2018b).

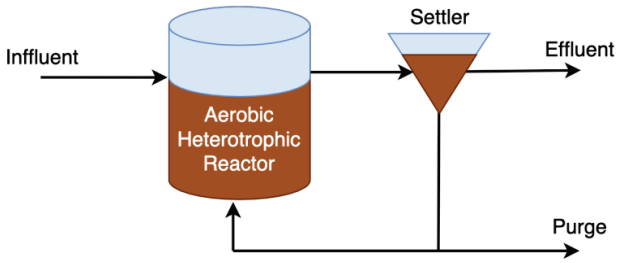

Fig. 2.1. Schematic representation of the continuous aerobic heterotrophic reactors.

Aeration was provided to the reactors ensuring oxygen concentrations between 3.5 and 7.5 mg O_2 L^{-1}. After a start-up period of a few days, the OMPs were spiked in the feeding at the desired concentrations, which were selected according to typical WWTPs influent concentrations (Table 2.1.).

The operation of the reactors was mainly monitored through the standard analytical methods (APHA, 2017) described in section 2.3. Soluble COD, ammonium, nitrate and nitrite concentrations, total suspended solids (TSS), VSS, pH and temperature were measured two to three times per week in the reactor influent (feedstock) and effluent. Measurements of OMPs and TPs were also performed when steady state conditions were reached at the different experimental conditions applied. Additionally, in **Chapter 5**, samples were taken for genomic and metatranscriptomic analysis.

2.2.2. Batch experiments

Batch assays were set to observe if variations in the heterotrophic microbial activity, determined as the maximum COD specific activity, affected OMPs biotransformation kinetics and, particularly, the k_{biol} (**Chapter 3**). The experiments were performed with COD initial concentrations of 0.2, 0.4, 0.6 and 0.8 g L^{-1} by varying the sodium acetate and acetic acid concentration and maintaining the micro and macronutrients used in the continuous reactor. The biomass was taken from the continuous reactors working at the respective organic loading rates (OLR) (**Chapter 3**) to ensure that the microbial population was adapted to the experimental conditions of the assays. For each batch, 18 flasks were prepared, three for each time point to have triplicates (0, 1, 3, 8, 24 and 48 h). At each of the mentioned times, 3 flasks were taken to determine the concentration of the OMPs. The VSS concentration was set in all cases to approximately 0.80 g VSS L^{-1} and the temperature and stirring were fixed at 25°C and 150 rpm (Innova 4300 Incubator Shaker – New Brunswick Scientific). Neutral pH was ensured using punctual additions of NaOH or HCl when necessary and an oxygen concentration above 4.5 mg O_2 L^{-1} was maintained during experimentation. To monitor the operation, the same analyses as in the continuous reactor (soluble COD, ammonium, nitrate and nitrite concentrations, TSS, VSS, pH and temperature) were conducted for each time point, except for the solids and nitrogen concentrations which were measured at times 0, 24 and 48 h. OMPs analysis was performed (solid and liquid phase) by taking samples from the flasks at each time point.

2.2.3. Sequential batch reactors

In **Chapter 5**, aiming to determine the key microbial and enzymatic players involved in SMX biotransformation, a total of 18 sequential batch reactors with a working volume of 100 mL were operated in an incubator at neutral pH, 25ºC, 150 rpm and with oxygen concentrations ranging between 3-5 mg O_2 L^{-1} for 25 days. Six different SMX concentrations were tested in triplicates: 0 (control), 50, 250, 500, 1000 and 2000 µg L^{-1}. The reactors were inoculated with activated sludge from the same WWTP as the continuous reactors and the operation was performed under sterile conditions.

The content of the sequential batch reactors was centrifuged daily at 6000 rpm and 10ºC for 10 min to separate the biomass from the supernatant. Then, the exhausted supernatant was removed and new feed was added together with the spike of SMX

corresponding to each reactor. Finally, the flasks were placed again in the incubator to resume operation. All the process was performed using a fume hood to ensure sterile conditions.

Ammonium, nitrate and nitrite concentrations, TSS and VSS were determined once per week, while the pH and acetate concentration were determined daily. Acetate was determined through gas chromatography using a DB-Wax-Agilent Technologies column (30mx0.250mmx0.25µm) while the other parameters were determined according to the standard analytical methods (APHA, 2017) described in section 2.3. Besides, samples from the feeding and the exhausted supernatant of the reactors were taken on days 2, 17 and 25 for SMX and TPs analysis. Finally, samples for proteomic (days 1 and 25) and 16S rRNA gene sequencing (day 25) analyses were also taken.

2.3. ANALYTICAL METHODS

Several analytical methods were applied to the experimental samples, generally according to Standard Methods (APHA, 2017).

2.3.1. Soluble chemical oxygen demand

The chemical oxygen demand (COD) is used to determine the concentration of the organic matter present in a liquid sample and is defined as the amount of oxygen required to oxidize the organic content. The COD was determined following a closed reflux titrimetric method based on the Standard Methods 5220C (APHA, 2017), but with minor modifications. Overall, the procedure uses a strong chemical oxidant (potassium dichromate) in an acid medium, along with a catalyst (silver sulphate) that improves the oxidation of some organic compounds. After digestion, the unreduced potassium dichromate is titrated with ferrous ammonium sulphate to determine its consumption. Then, using this value, the amount of oxidized organic matter present in the sample is calculated in terms of oxygen equivalents. The reaction is shown below:

$$Cr_2O_7{}^{2-} + \text{Organic matter} + H^+ \Longleftrightarrow Cr_3{}^+ + CO_2 + H_2O \qquad (1)$$

The required reagents, which allow for a detection range between 0.09 and 0.9 g COD L^{-1}, are listed below:

- Standard potassium dichromate digestion solution: 10.216 g of $K_2Cr_2O_7$ and 33 g of $HgSO_4$ are dissolved in 0.5 L of distilled water prior to the addition of

167 mL of concentrated H_2SO_4. Then, the solution is cooled down to room temperature and the solution is diluted to reach a total volume of 1 L.

- Sulphuric acid reagent (catalytic solution): 10.7 g Ag_2SO_4 are added to 1 L of concentrated H_2SO_4.
- Ferroin indicator solution: 1.485 g of $C_{18}H_{18}N_2 \cdot H_2O$ and 0.695 g of $SO_4Fe \cdot 7H_2O$ are dissolved in 0.1 L of distilled water.
- Potassium dichromate solution 0.05N: 1.226 g of $K_2Cr_2O_7$ are dissolved in 0.5 L of distilled water.
- Ferrous ammonium sulphate titrant (FAS) 0.035N: 13.72 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ are dissolved in distilled water before adding 20 mL of concentrated H_2SO_4 and dissolving to a total volume of 1 L.

Firstly, samples are filtered through a pore size filter of 0.45 µm (MF-Millipore, Millipore) to determine the soluble COD. Then, 2.5 mL of each sample are placed in 10 mL digestion glass vessels, to which 1.5 mL of the digestion solution and 3.5 mL of the sulphuric acid reagent are added slowly and carefully to avoid mixing of the reagents at this stage. Similarly, blanks are prepared using 2.5 mL of distilled water. All vessels are sealed with Teflon, tightly capped and mixed before placing them in the block digester (ECO 16, VELP Scientific) previously set at 150 ºC for two hours. After digestion, the tubes are cooled to room temperature and then each sample is transferred to a 50 mL Erlenmeyer flask which is magnetically stirred. Then, 1-2 drops of the ferroin indicator are added and the solution is titrated using a microburet with FAS until a change in color from light blue to red/orange is observed.

To determine the normality of the FAS solution, 5 mL of the potassium dichromate standard solution are mixed with 5 mL of distilled water and 3.5 mL of the catalytic solution. Then, after cooling down the sample to room temperature, titration is performed as previously explained.

Finally, the COD concentration is calculated taking into account the two equations below:

$$N_{FAS} = \frac{0.025}{V_{FAS}} \qquad (2)$$

$$COD\left(\frac{g}{L}\right) = \frac{(A-B)\cdot N_{FAS}\cdot 8}{V} \cdot DF \qquad (3)$$

Where N_{FAS} is the normality of the FAS solution (mol L^{-1}); V_{FAS} is the volume of the FAS solution consumed in the titration (mL); A and B are the volumes of the FAS solution consumed by the blank and the sample, respectively; V is the sample volume used before dilution; DF is the dilution factor applied to the sample and 8 stands for the conversion factor between COD and FAS (g COD mol^{-1} FAS).

2.3.2. Nitrogen compounds

All nitrogen analyses were performed after filtering the samples (0.45 µm, MF-Millipore, Millipore) to remove solid particles, according to the Standard Methods (APHA, 2017).

2.3.2.1. Nitrite

Nitrite concentration was determined spectrophotometrically using a method based on the production of a violet azo dye at a pH ranging between 2 and 2.5. It consists of a coupling between diazotized sulphanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride (NED dihydrochloride), as described in the Standard Methods (APHA, 2017) under the 4500-NO_2^- B procedure (Colorimetric method). The required reagents are:

- Sulphanilamide solution: 10 g of sulphanilamide ($C_6H_8N_2O_2S$) are dissolved in 0.1 L of concentrated HCl and 0.6 L of distilled water. When the solution is cool, additional distilled water is added until reaching a total volume of 1 L.
- NED solution: 0.5 g of NED ($C_{10}H_7NH(CH_2)_2NH_2 \cdot 2HCl$) are dissolved in 0.5 L of distilled water.

The determination procedure consists of the addition of 0.1 mL of each reagent to 5 mL of sample. Then, at least 20 minutes of reaction time are required before measuring the colored sample at a wavelength of 543 nm in the spectrophotometer (Shimadzu UV-1800).

The quantification is done with a calibration curve, which is prepared by making dilutions in the range of 0-0.25 mg NO_2^--N L^{-1} from a stock solution of 10 mg NO_2^- L^{-1}. Then, the absorbance of each sample is measured spectrophotometrically and related to the nitrite concentration (NO_2^--N L^{-1}) (Fig. 2.2.).

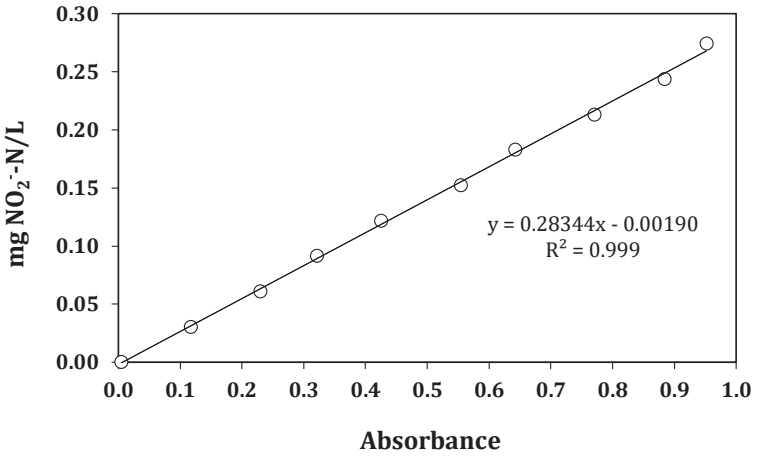

Fig. 2.2. Calibration curve for the determination of the concentration of nitrite.

2.3.2.2. Nitrate

Nitrate concentration was determined through measurements of UV absorption at the wavelengths of 220 and 275 nm, which allows avoiding errors caused by the absorbance of dissolved organic matter. The procedure corresponds to the 4500-NO_3^-B method listed in the Standard Methods (APHA, 2017). The required reagents are the following:

- HCl 1 N: 8.3 mL of HCl (37% w/v, 1.19 g L^{-1}) dissolved in 0.1 L of distilled water.
- Sulfamic acid: Commercial reagent (H_3NSO_3).

The determination procedure consists of adding 100 µL of HCl 1 N and a pinch of sulfamic acid, which allows avoiding interferences in the measurement caused by nitrite, to 5 mL of sample. Then, the absorbance is measured at 220 and 275 nm using the spectrophotometer (Shimadzu UV-1800) and the nitrate concentration (NO_3^--N L^{-1}) is calculated using the calibration curve (Fig. 2.3.). The absorbance related to nitrate is obtained according to Eq. 4.

$$\text{Absorbance } (NO_3{}^- - N) = Abs_{220} - 2 \cdot Abs_{275} \quad (4)$$

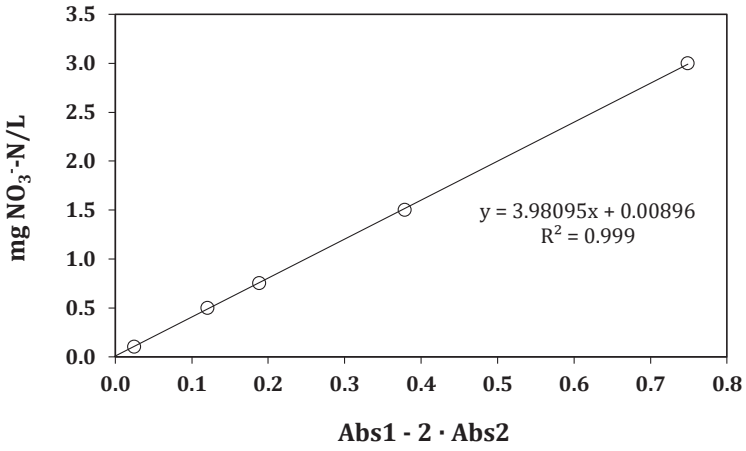

Fig. 2.3. Calibration curve for the determination of the concentration of nitrate.

The calibration curve is prepared using a 10 mg NO_3^- L^{-1} stock solution, which is diluted to obtain samples ranging between 0-3 mg NO_3^--N L^{-1}. Then, the absorbance is measured spectrophotometrically and related to the nitrate concentration.

2.3.2.3. Ammonium

A spectrophotometric methodology was selected to determine the concentration of nitrogen in the ammonium form. The analysis is based on the production of indophenol blue through the reaction of ammonia with salicylate and hypochlorite, under the presence of sodium nitroprusside (Bower and Holm-Hansen, 1980). Thus, the required reagents are the following:

- Reagent A: 0.28 g of sodium nitroprusside ($Na_2(Fe(CN)_5NO)$) and 440 g of sodium salicylate ($C_7H_5NaO_3$) dissolved in 1 L of distilled water.
- Reagent B: 18.5 g of NaOH and 120 g of sodium citrate ($Na_3C_6H_5O_7$) dissolved in 1 L of distilled water.
- Reagent C: 5% (w/w) commercial solution of sodium hypochlorite (NaClO).
- Reagent D: Solution consisting of a mixture of reagent B and Reagent C in a 7:1 ratio. This solution remains stable for 1 h after preparation.

The determination procedure consists of adding 600 µL of reagent A and 1 mL of reagent D to 5 mL of the previously filtered sample. Then, the mixture is left to react for 2 h in an environment protected from light. The measurement of the colored sample was

performed with a spectrophotometer (Shimadzu UV-1800) at a wavelength of 640 nm and the quantification was done with help of a calibration curve (Fig. 2.4.).

The calibration curve is prepared using a stock solution of 10 mg NH_4^+ L^{-1}, which was diluted in several samples in the range of 0-0.9 mg NH_4^+-N L^{-1}. Then, the absorbance of each sample was measured in the spectrophotometer and linked to the prepared ammonia concentration.

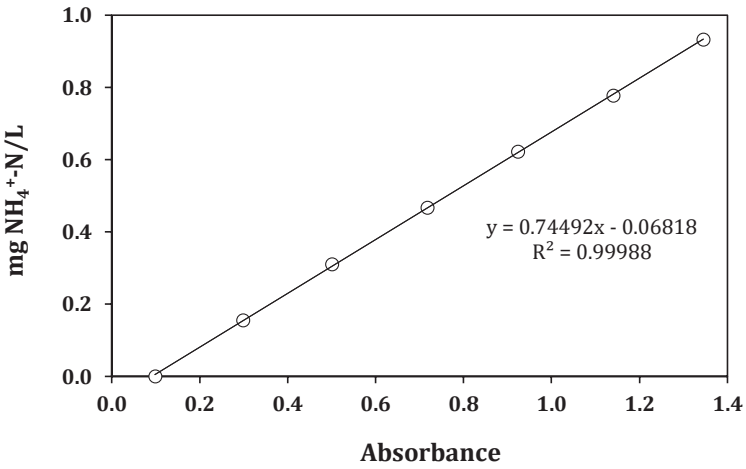

Fig. 2.4. Calibration curve for the determination of the concentration of ammonium.

2.3.3. Solids concentration

TSS and VSS were determined according to the methods 2540D and 2540E, respectively, detailed in the Standard Methods (APHA, 2017).

For the determination of the TSS, a well-mixed sample of the bioreactors is taken (aiming to yield a residue between 2.5 and 200 mg) and filtered through a weighted glass fiber filter (Whatman, GF/C, 4.7 cm of diameter, 1.2 µm of pore size), which needs to be dried before its use in the oven (around 15 min). The residue retained in the filter is dried in the oven at 105ºC until a constant weight is reached (approximately 2-3 h). The weight increase in the filter represents the TSS content in the sample, as shown in the following equation:

$$\text{Total suspended solids (g/L)} = \frac{A-B}{V} \qquad (5)$$

Where A represents the weight of the oven-dried filter, B the weight of its tare and V the volume of the liquid sample taken from the reactor.

The VSS is determined by transferring the dried filters, which allowed to calculate the TSS, to a muffle furnace for calcination at 550ºC for 20-30 min, until a constant weight is reached. The weight loss during ignition corresponds to the volatile fraction (Eq. 6), providing an estimate of the organic matter concentration present in the solid fraction of the bioreactors.

$$\text{Volatile suspended solids (g/L)} = \frac{A-C}{V} \qquad (6)$$

Where A stands for the weight of the oven-dried filter, C for the weight of the filter after calcination at 550ºC and V for the volume of the liquid sample taken from the reactor.

2.3.4. Dissolved oxygen, temperature and pH

The pH was measured in the reactors using an electrode (Crison Instruments GLP22, USA) equipped with an automatic compensatory temperature device connected to a pH-meter (GLP-22, Crison Instruments). The electrode was calibrated at room temperature with two standard buffer solutions of pH 7.00 and 4.00.

The dissolved oxygen was measured using a probe with a membrane-covered galvanic dissolved oxygen sensor (LDO101) connected to a digital multimeter device (HQ40D, HACH Lange, USA). This same probe was equipped with a thermopar that was used to take temperature measurements.

2.3.5. Organic Micropollutants

The determination of OMPs concentration in the liquid phase of the bioreactors begins with a centrifugation step at 3500 rpm for 10 min, while the feeding samples, which lack a solid fraction, do not need this step. Then, the supernatants and the feeding samples are prefiltered (AP4004705, Millipore) and filtered again at 0.45 µm (HAWP04700, Millipore). Lastly, solid-phase extraction (SPE) is performed with 200 mL samples and 60 mg Oasis HLB cartridges (Waters, Milford, MA, USA), as described by Fernandez-Fontaina et al. (2013). The quantification of antibiotics (ERY, ROX, SMX, TMP), neurodrugs (FLX, CBZ, DZP) and hormones (E1, E2, EE2) was performed using an Agilent G1312A liquid chromatography instrument with a binary pump and automatic

injector HTC-PAL (CTC Analytics) connected to a mass spectrometer API 4000 triple quadrupole (Applied Biosystems). Musk fragrances (HHCB, AHTN, ADBI), anti-inflammatories (IBP, NPX, DCF), xenoestrogens (BPA, OP, NP) and the biocide (TCS) were quantified using gas chromatography (Varian CP-3900) coupled to an ion trap spectrometer (Varian CG-2100). The quantification procedure was done according to previous studies (Alvarino et al., 2014). All OMPs analyses were performed in triplicate. Further information regarding the recoveries and limits of quantification and detection of the OMPs can be found in Table 2.4. Overall, the recoveries in the liquid matrix ranged between 70-95% and in the solid matrix between 50-60%, while the limits of quantification and detection ranged between 0.006-0.505 μg L^{-1} and 0.002-0.168 μg L^{-1}, respectively.

Table 2.4. Recoveries, limits of quantification and limits of detection for the studied OMPs.

OMPs	Recovery liquid phase (%)	Recovery solid phase (%)	Quantification limit (μg L^{-1})	Detection limit (μg L^{-1})
ERY	80%	50-60%	0.006	0.002
ROX	90%	50-60%	0.006	0.002
TMP	85%	50-60%	0.006	0.002
SMX	90%	50-60%	0.006	0.002
IBP	95%	50-60%	0.025	0.008
NPX	95%	50-60%	0.032	0.011
CBZ	85%	50-60%	0.505	0.168
DZP	90%	50-60%	0.505	0.168
ADBI	70%	50-60%	0.063	0.021
HHCB	70%	50-60%	0.063	0.021
AHTN	70%	50-60%	0.063	0.021
TCS	75%	50-60%	0.063	0.021
E1	90%	50-60%	0.012	0.004
E2	90%	50-60%	0.012	0.004
EE2	90%	50-60%	0.012	0.004

The solid phase of the bioreactor samples was frozen and lyophilized. To quantify the OMPs sorbed onto the aerobic sludge, ultrasonic solvent extraction (USE) was performed based on the procedure described by Ternes et al. (2005). The USE technique consisted of three sequential extractions with methanol and two with acetone applied to freeze-dried samples of approximately 0.5 g. During each extraction, the samples were sonicated for 15 min and centrifugated at 1500 rpm for 5 min. Then, the supernatants were combined, filtered through glass wool, evaporated (TurboVap LV, Biotage) flowing nitrogen (200 kPa, 30 °C) and resuspended in water. Finally, SPE and OMPs quantification was performed as described for the liquid phase.

2.3.6. Transformation product analysis and structure elucidation

To detect TPs, the bioreactor samples were analysed using reversed-phase liquid chromatography coupled to a high-resolution quadrupole Orbitrap mass spectrometer.

Full-scan MS spectra were acquired both in positive and negative ionization modes and the acquisition of MS2 fragmentation spectra was triggered at *m/z* values corresponding to a list of masses of suspected TPs. This list was compiled using previously found TPs in the literature, the EAWAG pathway prediction system (EAWAG-BBD, 2020) and by manually applying a range of plausible atomic modifications (hydroxylation, dihydroxylation, demethylation, dehydrogenation, hydrogenation, decarboxylation, etc.).

In **Chapter 4**, the suspect TP screening was performed using the software Compound Discoverer 3.1. (Thermo Scientific). TP candidate peaks had to fulfill the following criteria to be selected for further analysis:

1. Intensity above a certain threshold: maximum area > 25000.
2. The peaks had to have a reasonable peak shape.
3. If a chemical formula was proposed, the observed isotopic pattern had to match the theoretical one.
4. If a MS2 spectrum was available, the fragmentation prediction and the matches to the fragments of the respective parent compound were taken into account. Besides, the FISh scoring node of Compound Discoverer 3.1. was used, rejecting compounds with a score below 50.
5. The peak area of the TPs had to be at least 10 times higher in the effluent than in the influent of the reactor.

The confidence in the TPs differed depending on the availability of structural evidence. To communicate the confidence in the structural interpretation, confidence levels were assigned as proposed by Schymanski et al. (2014): Level 5 (exact mass), Level 4 (unequivocal molecular formula), Level 3 (tentative candidates), Level 2 (probable structure) and Level 1 (confirmed structure). TP evidence, i.e., observed changes in molecular formula and structure, was used to assign corresponding reaction types to the OMPs.

Differently, in **Chapter 5**, the TP screening was performed by the Center of Mass Spectrometry and Proteomics belonging to the University of Santiago de Compostela with the aid of the software TASQ (Bruker).

2.3.7. DNA

In **Chapter 5**, genomic DNA was extracted using the Nucleospin Microbial DNA extraction kit (Machery-Nagel) from homogenized 1 mL samples taken from the continuous bioreactor at the end of each stage and all sequential batch reactors on day 25.

Quantification and quality control were performed with Nanodrop and Qubit fluorometer (Thermo Fisher Scientific Waltham) and the analyses were carried out by AllGenetics & Biology SL (www.allgenetics.eu) in an Illumina PE150 platform.

The bioinformatic analysis was performed using the Microbial Genomics module (version 21.1) workflow of the CLC Genomics workbench (version 21.0.3). Raw sequences were filtered to remove low-quality reads, clustered into Operational Taxonomic Units (OTUs) at 97% cutoff for sequence similarity and classified against the non-redundant version of MiDAS 4 (Dueholm et al., 2021) and SILVA SSU (release 132; http://www.arb-silva.de) reference taxonomy databases. Only the most abundant bacterial OTUs (above 1 % of the total observed OTUs) were considered.

2.3.8. Proteomics

Proteomic analyses were carried out in **Chapter 5** aiming to identify the key enzymes involved in the biotransformation of SMX.

2.3.8.1. Proteome extraction

Proteome extractions were performed to 1 mL homogenized samples belonging to the inoculum and the sequential bath reactors on day 25. Cells were harvested by centrifugation at 6000 rpm, washed twice with PBS buffer and subjected to 90 ºC digestion for 20 minutes in 1% SDS Tris-HCl extraction buffer. Then, the cells were broken by performing a 12-minute beating with glass beads in a cell disruptor and centrifugation at 3000 rpm was applied to remove cell debris and glass beads. Proteins were then precipitated with acetone in two consecutive steps at -20ºC and further resuspended with molecular grade water. The protein concentration was estimated through a BCA assay at 540 nm upon calibration with a BSA standard curve. Finally, triplicate samples were pooled together to obtain a mixed sample corresponding to the inoculum and each SMX concentration studied. The quality of the proteome samples was confirmed using SDS-PAGE with 4-12 % Bis-Tris acrylamide NuPAGE gels (Thermo Fischer).

2.3.8.2. Protein detection

To perform in solution shotgun metaproteomics (Y. Zhang et al., 2013), the samples were reduced, alkylated, trypsin-digested and acidified. Then, the digested samples were desalted, vacuum-dried and reconstituted in water with 2% acetonitrile and 0.1% formic

acid. The peptide mixtures (200 ng) were analysed in a nanoElute (Bruker Daltonics) nano-flow liquid chromatograph equipped with a C-18 reversed-phase column coupled to a high-resolution TIMS-QTOF (timsTOF Pro, Bruker Daltonics) with a CaptiveSpray ion source (Bruker Daltonics). After ESI ionization, the peptides were analyzed in data-dependent mode with the Parallel Accumulation–Serial Fragmentation (PASEF) enabled.

2.3.8.3. Protein data analysis

Mass spectrometry raw files were processed with PEAKS Studio 10.6 build 20201221 (Bioinformatics Solutions Inc.). The MS/MS spectra were matched to in silico derived fragment mass values of tryptic peptides against the UniProtKB/Swiss-Prot database (release 2021_02).

The inoculum samples were analyzed independently, while samples taken during the experimental procedure were analyzed as a batch using the "Label-free" semi quantification module from PEAKS. The Spec value, which is based on peptide spectrum matches, was used as an indicator for the relative abundance of the proteins in each sample (Ma et al., 2003). The proteins identified with less than 2 unique peptides were excluded from the analyses and, for the taxonomic approach, the proteins were grouped at the genus level. The obtained list of peptide sequences was additionally processed with the UniPept Desktop v.1.2.1. for molecular function categorization. Aiming to explore if an association could be made between some enzymes and SMX biotransformation, the list of identified proteins was analyzed trying to: *i)* search for enzymes previously linked to sulfonamide biotransformation in the literature and *ii)* search for overexpressed enzymes at increased SMX concentrations.

2.4. CALCULATIONS

Several calculations and mass balances applied during the operation of the bioreactors are presented in this section.

2.4.1. Calculations of reactor performance

Several indicators were calculated to characterize the performance of the continuous reactors, including the net activated sludge produced, the endogenous biomass decay rate, the nitrogen oxidized, the recirculation ratio and the oxygen requirements.

The net waste activated sludge produced was estimated assuming typical heterotrophic parameters of biomass yield (Y = 0.4 $g_{biomass}$ $g_{substrate}^{-1}$), endogenous decay coefficient (k_d = 0.1 d^{-1}) and cell debris fraction (f_d = 0.15) (Metcalf & Eddy, 2014), as shown in Eq. 7:

$$P_{X,VSS} = \frac{Y\,F\,(COD_{inf} - COD_{eff})}{1+(k_d)\,SRT} + \frac{(f_d)\,(k_d)\,Y\,F\,(COD_{inf} - COD_{eff})\,SRT}{1+(k_d)\,SRT} \quad (7)$$

The endogenous decay rate of biomass in the reactor was also estimated, as indicated in Eq. 8:

$$rd = -kd \cdot X_{VSS} \quad (8)$$

Moreover, as shown below (Eq. 9), the oxidized nitrogen was calculated:

$$N_{ox} = TKN_0 - N - \frac{0.12 P_{X,VSS}}{F} \quad (9)$$

Where N_{ox} (mg L^{-1}) is the nitrogen oxidized, TKN_0 (mg L^{-1}) is the influent total Kjeldahl nitrogen and N is the effluent N-NH_4^+ concentration (mg L^{-1}).

To maintain a constant biomass concentration in the reactor, purge was performed daily. Besides, biomass recirculation was performed in a ratio close to 1, typical for activated sludge systems (Metcalf & Eddy, 2014), as indicated in Eq. 10:

$$R = \frac{1-\left(\frac{HRT}{SRT}\right)}{\left(\frac{X_R}{X_{VSS}}\right)-1} \quad (10)$$

Where X_R (g L^{-1}) is the biomass concentration in the settler.

To ensure aerobic conditions, the dissolved oxygen concentration was controlled. Experimental values over 3 mg O_2 L^{-1} were always maintained in the reactor, following the guidelines of Metcalf and Eddy (2014) for aerobic biological systems during wastewater treatment. The oxygen provided was sufficient to fully oxidize the influent COD (Eq. 11).

$$R_0 = F(COD_{eff} - COD_{inf}) - 1.42 P_{X,VSS} = 1.8\ g\ O_2\ d^{-1} \quad (11)$$

2.4.2. OMPs mass balance

The removal of the OMPs in the experiments was associated with three mechanisms: volatilization, sorption to the sludge and biotransformation. The overall mass balances in the reactors are expressed as shown in the following equations (Eq. 12 and 13):

$$\frac{dC_W}{dt} = \frac{F_{in}(t)}{V} - \frac{F_{out}(t)}{V} - \left(\frac{dC_W}{dt}\right)_{volatilization} - \left(\frac{dC_W}{dt}\right)_{sorption} + \left(\frac{dC_W}{dt}\right)_{desorption} - \left(\frac{dC_W}{dt}\right)_{biotransformation} \quad (12)$$

$$\frac{dC_S}{dt} = \frac{F_{in}(t)}{V} - \frac{F_{out}(t)}{V} + \left(\frac{dC_W}{dt}\right)_{sorption} - \left(\frac{dC_W}{dt}\right)_{desorption} \quad (13)$$

Where C_W (µg L^{-1}) is the dissolved OMPs concentration, C_s (µg L^{-1}) is the OMPs concentration in the solid phase, F_{in} (µg d^{-1}) is the inlet OMPs flow rate, F_{out} (µg d^{-1}) is the outlet OMPs flow rate and V (L) is the bioreactor volume.

Volatilization is based on the transfer due to an equilibrium process between the liquid and gas phase and depends mostly on the physicochemical properties of the OMPs and the operating conditions of the process. In an activated sludge process, volatilization can occur due to stripping, which is controlled by the air flow, the OMPs concentration and the Henry's constant, and due to surface volatilization, which usually is not taken into account as a result of its lower relevance (Ltd I C Consultants, 2001). Volatilization can be neglected when a compound has a Henry constant below 10 Pa m^3 mol^{-1} and its Hc/K_{ow} (Henry's constant divided by the octanol-water partition coefficient) is lower than 10^{-4} (Ltd I C Consultants, 2001). This is the case of the OMPs studied in this thesis (Table 2.1.).

Sorption is based on the transfer due to an equilibrium process between the liquid and solid phase, including dissolved and colloidal organic matter, where OMPs simultaneously undergo sorption and desorption phenomena (Barret et al., 2010). Equilibrium is assumed to be reached generally in a matter of hours and depends on the OMPs physicochemical properties (including hydrophobicity, cation exchange, cation bridging, surface complexation and hydrogen-bonding (Plósz et al., 2010)), sludge quality and operating conditions (Pomiès et al., 2013). Two mechanisms are involved in the sorption process: *(i)* absorption, occurring due to hydrophobic interactions between the aliphatic and aromatic groups of the OMPs and the lipophilic cell membrane and fat fractions of the microorganisms and *(ii)* adsorption, happening due to electrostatic interactions of the positively charged groups of the OMPs and the negatively charged surfaces of the microbial population (Sipma et al., 2010). Since OMPs are present at low

concentrations in WWTPs, a linear model is normally assumed to describe this phenomenon (Pomiès et al., 2013), as shown below (Eq. 14 and 15):

$$\left(\frac{dC_W}{dt}\right)_{sorption} = k_{sor} \cdot C_W \cdot X_{TSS} \quad (14)$$

$$\left(\frac{dC_W}{dt}\right)_{desorption} = k_{desor} \cdot C_S \quad (15)$$

Where k_{sor} is the sorption kinetic constant (L g^{-1} d^{-1}), k_{desor} is the desorption kinetic constant (d^{-1}) and X_{TSS} is the total suspended solids concentration (g L^{-1}).

The partition coefficient (K_d), shown in Eq. 16, is the ratio between the kinetic constants at equilibrium conditions:

$$K_d = \frac{k_{sor}}{k_{desor}} = \frac{C_S}{C_W \cdot X_{TSS}} \quad (16)$$

Biotransformation consists of the biological removal of the compounds, which is typically calculated for the dissolved OMPs (Pomiès et al., 2013). Pseudo-first order kinetics are typically used to model the OMPs biotransformation rate (r_{biol}), represented in Eq. 17 (Schwarzenbach et al., 2003):

$$\left(\frac{dC_W}{dt}\right)_{biotransformation} = r_{biol} = k_{biol} \cdot C_W \cdot X_{VSS} \quad (17)$$

The biodegradation kinetic constants were calculated performing mass balances to the batch experiments (**Chapter 3**), as shown in Eq. 18:

$$k_{biol}\ (L\ g^{-1}\ d^{-1}) = \frac{C_{W0} - C_{Wt} \cdot (1 + k_d \cdot X_{TSS})}{C_{Wt} \cdot X_{VSS} \cdot t} \quad (18)$$

Where C_{w0} (µg L^{-1}) is the dissolved OMPs concentration at time 0 and C_{wt} (µg L^{-1}) is the dissolved OMPs concentration at time t, where the slope is maximum.

The biotransformation yield (Eq. 19) and the specific biotransformation rate (Eq. 20) were calculated to describe the behavior of the OMPs. The yield determines the biotransformation efficiency of the compounds and the biotransformation rate provides information regarding the cometabolic behavior of the OMPs and the performance of the reactor.

$$\text{Biotransformation yield (\%)} = \frac{F_{in} - F_{wout} - F_{sout}}{F_{in}} \cdot 100 \qquad (19)$$

$$\text{Specific biotransformation rate } (\mu g\ g_{VSS}^{-1}\ d^{-1}) = \frac{F_{in} - F_{wout} - F_{sout}}{X_{VSS} \cdot V} \cdot 100 \qquad (20)$$

Where F_{wout} (µg d^{-1}) is the outlet dissolved OMPs flow rate and F_{sout} (µg d^{-1}) is the outlet OMPs flow rate in the solid phase.

The outlet OMPs flow rate in the solid phase (F_{sout}) is calculated as shown in Eq. 21:

$$F_{sout} = C_S' \cdot X_{TSS-effluent} \cdot F_{effluent} + C_S' \cdot X_{TSS-purge} \cdot F_{purge} \qquad (21)$$

Where C_S' (µg g^{-1}) is the OMPs concentration in the solid phase, $F_{effluent}$ (L d^{-1}) is the flow of the effluent and F_{purge} (L d^{-1}) is the flow of the purge.

In **Chapter 3**, to determine if the yields and specific biotransformation rates of the OMPs were statistically different at the tested primary substrate specific biodegradation rates, R software 3.6.2. was used. The statistical tests were performed at a 5% significance level ($p < 0.05$).

CHAPTER 3. The organic loading rate affects organic micropollutants' cometabolic biotransformation kinetics under heterotrophic conditions in activated sludge

SUMMARY

Organic micropollutants (OMPs) are biotransformed cometabolically in activated sludge systems. However, the individual role that heterotrophs play is still not clear and there is still a gap regarding the influence of the heterotrophic activity on the cometabolic biotransformation kinetics and yield of the OMPs. To answer these questions, experiments with increasing primary substrate concentrations were performed under aerobic heterotrophic conditions in a continuous stirred tank reactor operated at several organic loading rates (OLR) with fixed hydraulic retention time. Moreover, the individual kinetic parameters were determined in batch assays with different initial substrate concentrations using the sludges from the continuous reactor. A set of 15 OMPs displaying a variety of physicochemical properties were spiked to the feeding in the ng L^{-1} - µg L^{-1} range. Results reveal that the biodegradation of the primary carbon source and the biotransformation of the OMPs occur simultaneously, in evidence of cometabolic behaviour. Moreover, the OMPs biotransformation kinetic constant (k_{biol}) shows a linear dependence with the OLR of the primary substrate for most compounds, suggesting that the heterotrophic activity affects the OMPs biotransformation kinetics. However, under typical activated sludge systems operating conditions (hydraulic retention times above 8 h), the biotransformation yield would not be significantly affected.

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Further information can be found in the "List of publications" (page 145).

3.1. INTRODUCTION

To characterize OMPs biotransformation, a pseudo-first order kinetic model is commonly assumed and the resulting biotransformation kinetic constant (k_{biol}) largely depends on the physicochemical properties of each particular pollutant (Lema and Suarez, 2017). In fact, Falås et al. (2016) concluded that k_{biol} varies more depending on the nature of the OMPs than on the experimental conditions. However, k_{biol}, as a kinetic constant, is also affected by the reactor operating conditions (such as temperature, pH and oxidation reduction potential), the presence and availability of co-substrates and the biochemical versatility of the sludge, as several studies demonstrate (Barceló, 2012; Petrovic et al., 2013). For instance, Alvarino et al. (2016) reported k_{biol} values of 0.09 and 0.05 L g VSS^{-1} d^{-1} for SMX under aerobic heterotrophic and autotrophic denitrifying conditions, respectively; Gulde et al. (2014) reported k_{biol} increments of one order of magnitude for atenolol at pH 8 compared to pH 6 under activated sludge conditions and Li et al. (2005) reported that E2 increased its k_{biol} value from 1.8 to 3.3 L g VSS^{-1} h^{-1} when increasing the temperature from 20 to 35ºC in batch experiments with activated sludge. Nonetheless, conversely, there are so far no conclusive studies regarding the influence of the OLR and the heterotrophic activity on k_{biol}.

The role of the OLR in the removal of the OMPs is, in fact, a topic of current and general interest in biological systems, but results still do not show a clear trend. For instance, Kora et al. (2020) showed that increasing the OLR improved the removal of 3 out of 5 OMPs spiked in a methanogenic-aerobic moving bed biofilm reactor and Moya-Llamas et al. (2018) found better biodegradations for 6 OMPs at higher OLRs in a UASB reactor coupled to a MBR. On the contrary, in a moving biofilm bed reactor, Abtahi et al. (2018) reported for some OMPs a maximum removal at the highest OLR tested and for others at the lowest OLR and Carneiro et al. (2020) reported a negative impact of increasing OLRs in the biodegradation of citalopram and SMX in anaerobic fix bed biofilm reactors. Finally, differently from these studies, Gonzalez-Gil et al. (2018) found no correlation between variations in the OLR and the biotransformation of most OMPs in methanogenic digesters.

The objective of the present chapter is to extend the knowledge behind OMPs biotransformation processes under exclusively aerobic heterotrophic conditions. More specifically, research is focussed on assessing the OMPs cometabolic biotransformation,

aiming to determine the relationship between the intensity of the heterotrophic activity and the OMPs biotransformation rate, with a particular focus on evaluating the effect on k_{biol}. The implications of understanding such linkage could be of great importance to design biological systems able to maximize OMPs removal. To reach these goals, a series of experiments in a continuous stirred tank reactor operated at different OLRs were performed, as well as batch experiments set with different initial COD concentrations.

3.2. MATERIALS AND METHODS

An aerated 5 L continuous stirred tank reactor connected to a 2 L settler (section 2.2.1.) set at an HRT of 1 d and a SRT around 5 d was inoculated with activated sludge at a concentration of 1 g L^{-1}. It was operated at four different OLRs (0.2, 0.4, 0.6 and 0.8 g COD L^{-1} d^{-1}, that resulted in 0.4, 0.6, 1.1 and 2.7 g COD g VSS^{-1} d^{-1}), selected trying to cover typical conditions of activated sludge WWTPs (Lema and Suarez, 2017; Metcalf & Eddy, 2014). Each experimental stage was maintained for one month and the OLR was changed by varying the organic matter concentration in the feeding while keeping constant the HRT. The feeding was based on acetate as organic carbon source (section 2.1.2.) and contained ERY, ROX, TMP, SMX, IBP, NPX, CBZ, DZP and TCS at concentrations of 10 µg L^{-1}; E1, E2 and EE2 at 1 µg L^{-1} and ADBI, HHCB and AHTN at 40 µg L^{-1} (section 2.1.1). Once steady state was reached at each OLR, inlet and outlet OMPs concentrations were measured (solid and liquid phase) by taking three samples from the feeding and the reactor vessel in three consecutive days.

Besides, analysis of the influent and effluent of the reactor was performed to determine the typical operational parameters according to the standard analytical methods described in **Chapter 2**. The soluble COD, ammonium, nitrate and nitrite concentrations, SSV, pH and temperature were measured two to three times per week.

Additionally, 4 batch assays using the same group of OMPs were set to analyze their biotransformation kinetics and k_{biol}. The experiments were aerated and performed at neutral pH, 25ºC, 150 rpm and COD initial concentrations of 0.2, 0.4, 0.6 and 0.8 g L^{-1} by varying the organic carbon concentration while maintaining the same micro and macronutrients used in the heterotrophic reactor. The inoculum was taken from the continuous reactors working at the respective OLRs, aiming at an initial concentration of 0.80 g VSS L^{-1}. For each batch assay, 18 flasks were prepared, three for each time point to have triplicates (0, 1, 3, 8, 24 and 48 h). At the mentioned times, 3 flasks were taken

to determine the concentration of the OMPs in the solid and liquid phases. The same conventional operational parameters as in the continuous reactor were determined at each time point, except for the solids and nitrogen concentrations which were measured at times 0, 24 and 48 h.

3.3. RESULTS AND DISCUSSION

3.3.1. Biotransformation yield

Fig. 3.1 shows the biotransformation yield, i.e. the biotransformation percentage, of the selected OMPs at the 4 OLRs in the aerobic heterotrophic reactor. The biotransformation extent varied considerably among OMPs, being their behaviour characterized as *(i)* low biotransformation (below 20%) for CBZ and DZP, *(ii)* medium-low biotransformation (20-50%) for TMP, *(iii)* medium-high biotransformation (50-80%) for ERY and *(iv)* high biotransformation (over 80%) for ROX, SMX, IBP, NPX, TCS, ADBI, HHCB, AHTN, E1, E2 and EE2. These results are consistent with literature information (Alvarino et al., 2018b; Fernandez-Fontaina et al., 2016).

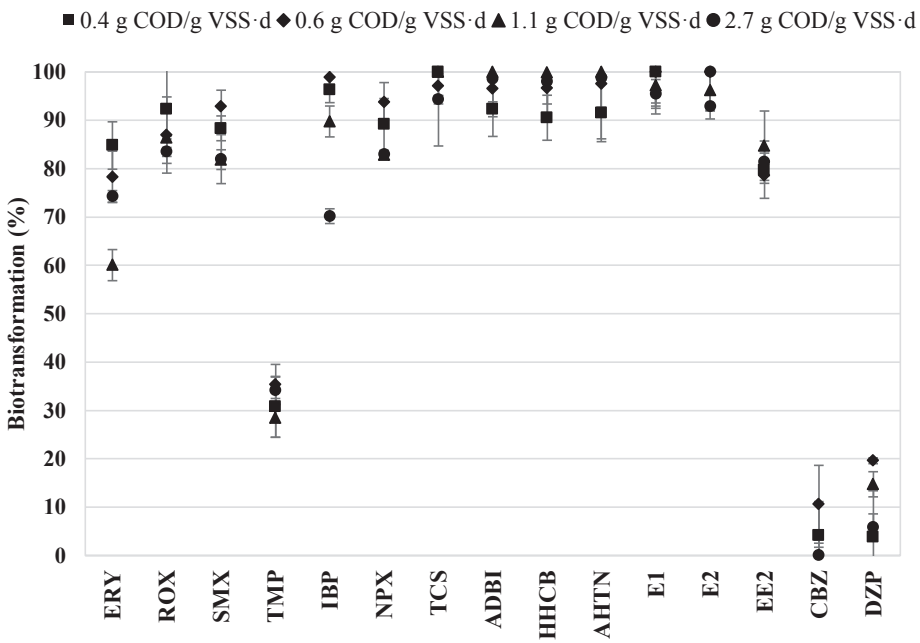


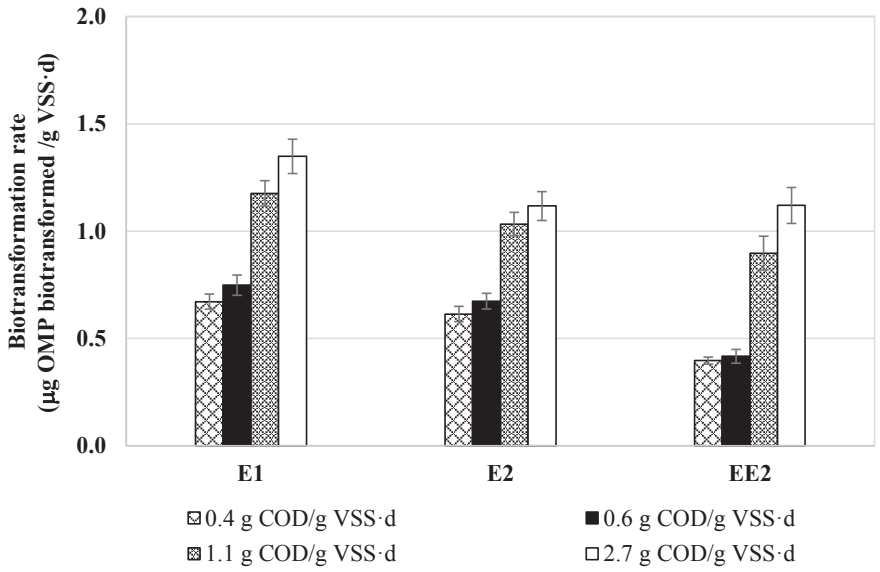
Fig. 3.1. OMPs biotransformation yield achieved in the aerobic heterotrophic reactor operated with fixed HRT (1 day) at 4 different specific OLRs of the primary substrate.

It can be observed that the biotransformation yield of most OMPs remained constant throughout the different OLRs (biotransformation differences below 10 percentage points and not statistically significant; $p > 0.05$). Only one value for ERY (experiment with 1.1 g COD g VSS^{-1} d^{-1}) and another for IBP (experiment with 2.7 g COD g VSS^{-1} d^{-1}), significantly ($p < 0.05$) deviated (between 15-20 percentage points) from the values of the other three conditions. These events for ERY and IBP cannot be attributed to a specific trend or behaviour. Therefore, overall, the results of Fig. 3.1 indicate that increasing the aerobic heterotrophic cometabolism did not affect the OMPs biotransformation yield under the conditions tested. Several hypotheses could explain this behaviour. The first one is that the enzymes involved in the OMPs biotransformation might not be increasingly stimulated at higher OLRs. According to Stadman (1970), in catabolic pathways, enzymes can be classified as "constitutive" enzymes, whose concentration is independent of the presence of their substrates, and "inducible" enzymes, that are produced when their immediate substrates or suitable derivatives are present. If the enzymes involved in the biotransformation of the OMPs belong to the first class and were not increasingly stimulated, the yield could have remained stable even at higher OLRs. A second hypothesis is that the maximum cometabolic rate towards OMPs is already reached at the lowest OLR and, therefore, increasing the degradation rate of the primary substrate (acetate) does not have an effect on the OMPs. In this regard, it is commonly assumed that the oxidation rate of a non-growth substrate (OMPs) should always be linked to the oxidation rate of a growth substrate (acetate) proportionally; however, some studies have shown that this is not necessarily always the case (Criddle, 1993; M. H. Kim et al., 2020). Thirdly, the OMPs may have already achieved their biotransformation limits due to thermodynamic constraints. This event could be caused by reversibility of the biological reactions, leading to a chemical equilibrium between the parent compound and the TPs, as previously suggested (Gonzalez-Gil et al., 2019a). Lastly, the primary metabolism may increase the specific biotransformation rate, but the HRT may have been high enough to hide this effect, showing the same biotransformation yield in all cases. In common biological treatments, as in activated sludge, the optimal HRT for OMPs removal is 24 h or longer (Boonnorat et al., 2019). Nevertheless, it has been proven that lower times may be sufficient to achieve the maximum biotransformation extent for several compounds (Boonnorat et al., 2019; Ejhed et al., 2018). Overall, the results shown in Fig. 3.1 are insufficient to determine which of these four hypotheses is more likely, being necessary to evaluate the behavior of the biotransformation rate.

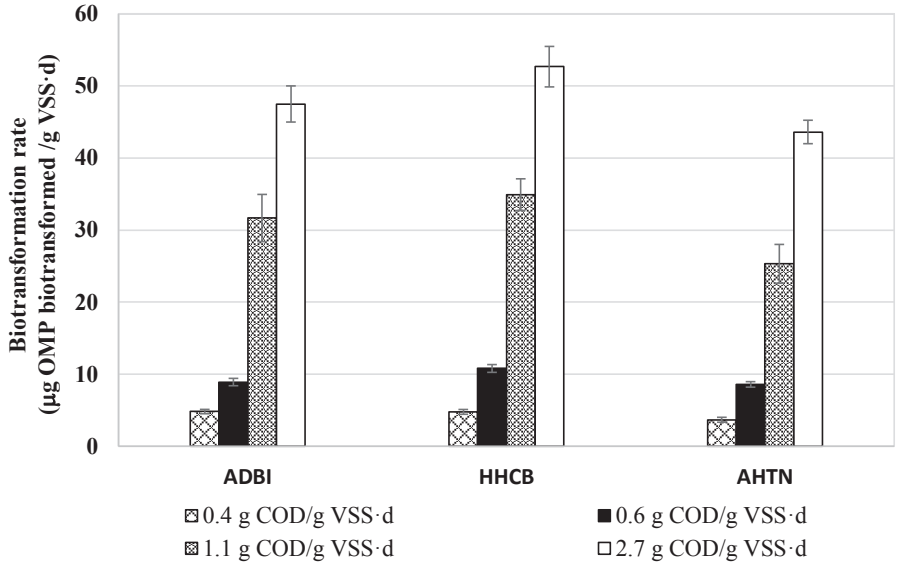
3.3.2. Specific biotransformation rate

In Fig. 3.2, it can be observed the direct relationship between the OMPs biotransformation rate and the specific sludge activity: the higher the specific biodegradation rate of the growth substrate, the higher the OMPs specific biotransformation rate. The dependence of the biotransformation rate of the secondary substrates (OMPs) with the biodegradation of the primary substrate (acetate) is clear proof of cometabolism (Lema and Suarez, 2017). When the concentration of the primary substrate is increased (i.e., a higher OLR is applied), a higher microbial activity of the biomass is achieved. Hence, if an increment of the OMPs biotransformation rate also occurs, it indicates that the OMPs are been cometabolized by the same enzymes involved in the metabolism of the growth substrate. In Fig. 3.2, the differences in the specific biotransformation rates were statistically significant ($p < 0.05$) for all experimental conditions and all OMPs tested.

A)



B)



C)

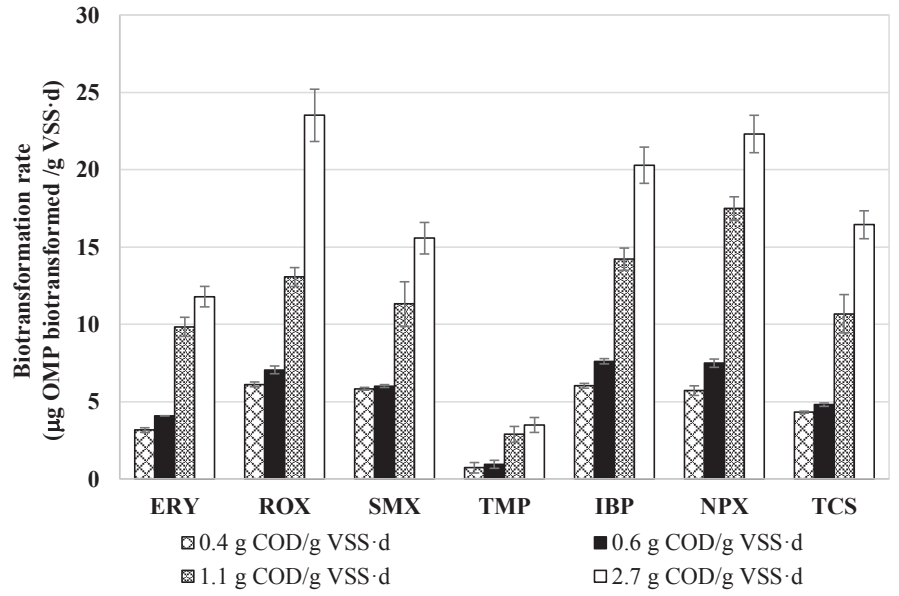


Fig. 3.2. Specific OMPs biotransformation rate (µg OMP g VSS^{-1} d^{-1}) at 4 specific biodegradation rates of the primary substrate (g COD g VSS^{-1} d^{-1}). A) OMPs fed at 1 µg L^{-1} d^{-1}. B) OMPs fed at 40 µg L^{-1} d^{-1}. C) OMPs fed at 10 µg L^{-1} d^{-1}.

Despite all OMPs following a cometabolic biotransformation trend, there are some differences between the compounds, which do not show a homogeneous impact of the OLRs in their biotransformation rates. For instance, while the biotransformation rate of TCS or SMX only slightly increases between the conditions of 0.4 g COD g VSS^{-1} and 0.6 g COD g VSS^{-1}, other compounds such as ADBI, HHCB and AHTN are much more affected. The explanation for this variability could be related to the enzymatic biotransformations. It is very likely that the OMPs are biotransformed by different enzymes present in the heterotrophic metabolism and that their affinity for such enzymes varies, leading to changes in their cometabolic biotransformation rates. In fact, according to M. H. Kim et al. (2020), the cometabolic biotransformation is influenced by the ratio of the initial growth to non-growth substrate concentration and the specificity constant (the kinetic efficiency, which measures how efficiently an enzyme converts substrates into products) ratio of the growth and non-growth substrates, which could be different for each compound.

Overall, the specific OMPs biotransformation rates at 2.7 g COD g VSS^{-1} d^{-1} were around 2.5- and 4-times fold the values obtained in the experiment performed at 0.4 g COD g VSS^{-1} d^{-1}. Nevertheless, it is noticeable that in comparison to the other compounds, ERY, TMP, E1, E2 and EE2 (Fig. 3.2) showed reduced rate increments between the experiments performed at 1.1 and 2.7 g COD g VSS^{-1} d^{-1} (although still significantly different ($p < 0.05$)). These lower changes at the higher OLRs could indicate that those compounds are close to reaching a maximum cometabolic biotransformation rate (Gonzalez-Gil et al., 2018b), likely determined by some thermodynamic constraints, as suggested in the third hypothesis of section 3.1. In any case, Fig. 3.2 shows that neither the first hypothesis (enzymatic stimulation was not occurring at higher OLRs) nor the second (the maximum cometabolic rate had been achieved at the lowest OLR) were taking place. Therefore, the best explanation for the results of Fig. 3.1 is that, although the biotransformation rates varied with the OLR, the HRT was high enough to hinder an effect on the biotransformation yield.

Previous studies have also proven OMPs cometabolism in different environments. For example, Fernandez-Fontaina et al. (2012) reported a cometabolic effect in the biotransformation of IBP, ERY, ROX and AHTN, among others, in nitrifying reactors, likely due to the action of AMO. Similarly, Majewsky et al. (2011) determined that the heterotrophic active fraction was correlated with the removal extent of pharmaceuticals

such as DCF and SMX. Interestingly, under anaerobic conditions, González-Gil et al. (2018) reached a methanogenic activity high enough not to show a relationship with the biotransformation rate of most OMPs, contrary to previous anaerobic studies with lower activities of the primary substrate (Alvarino et al., 2014).

The cometabolic effect proved in this chapter might imply that reactors operating at higher OLRs could have improved performance. Higher OLRs are often related to increased microbial activity and to a larger expression or activity of the enzymes involved in the metabolism of the primary substrates, leading to higher catalytic activity in the reactor which could enhance the cometabolic biotransformation of the OMPs. Therefore, high load WWTPs are likely to have a better performance in OMPs biotransformation by increasing their yield or, if a limitation has been reached (as in Fig. 3.1), by being able to achieve the same biotransformation yield in a shorter amount of time than low load WWTPs, allowing operations at shorter HRTs.

3.3.3. Influence of heterotrophic activity on the OMPs biotransformation constant

In previous sections, it was shown that a higher heterotrophic activity leads to a higher specific OMPs biotransformation rate, indicating that heterotrophs are key on r_{biol}. Based on Eq. 17 (section 2.4.2.), it seems that the influence of the heterotrophic activity on r_{biol} occurs through changes in k_{biol}. Thus, batch tests were conducted to determine the correlation between the microbial activity and k_{biol}, as well as between the removal of the primary substrate and the OMPs.

Studying the interaction between the OMPs and the growth substrates can help to understand their dynamics and control the fate of the OMPs by properly managing the primary substrates. As shown in Fig. 3.3, the biotransformation of the OMPs (exemplified for ROX, SMX, NPX and HHCB) occurs simultaneously with the degradation of the primary carbon source (acetate). This finding agrees with the common cometabolic biotransformation theory, where the OMPs, induced by the presence of the primary substrate, enter its metabolic pathway and undergo simultaneous biodegradation (King et al., 1997). OMPs do not yield enough energy to support microbial growth and their biotransformation occurs fortuitously when their chemical structure is modified thanks to the enzymatic action that degrades the primary substrate. Similar to the findings shown in Fig. 3.3, Aeming et al. (2019) determined that the removal of 4 endocrine disruptors (fluoranthene, benzo(b)fluoranthene, benzo(a)pyrene and NP) had a synchronal fate to

the organic matter during anaerobic digestion and composting. According to the results shown in Fig. 3.2 and Fig. 3.3, it is proved that the main driver during the heterotrophic biotransformation of OMPs is the presence of the growth substrate due to cometabolism.

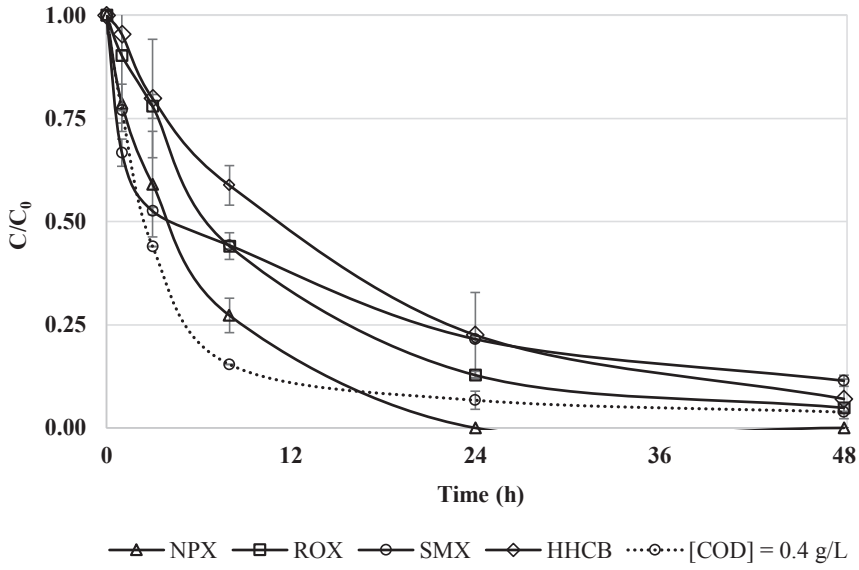
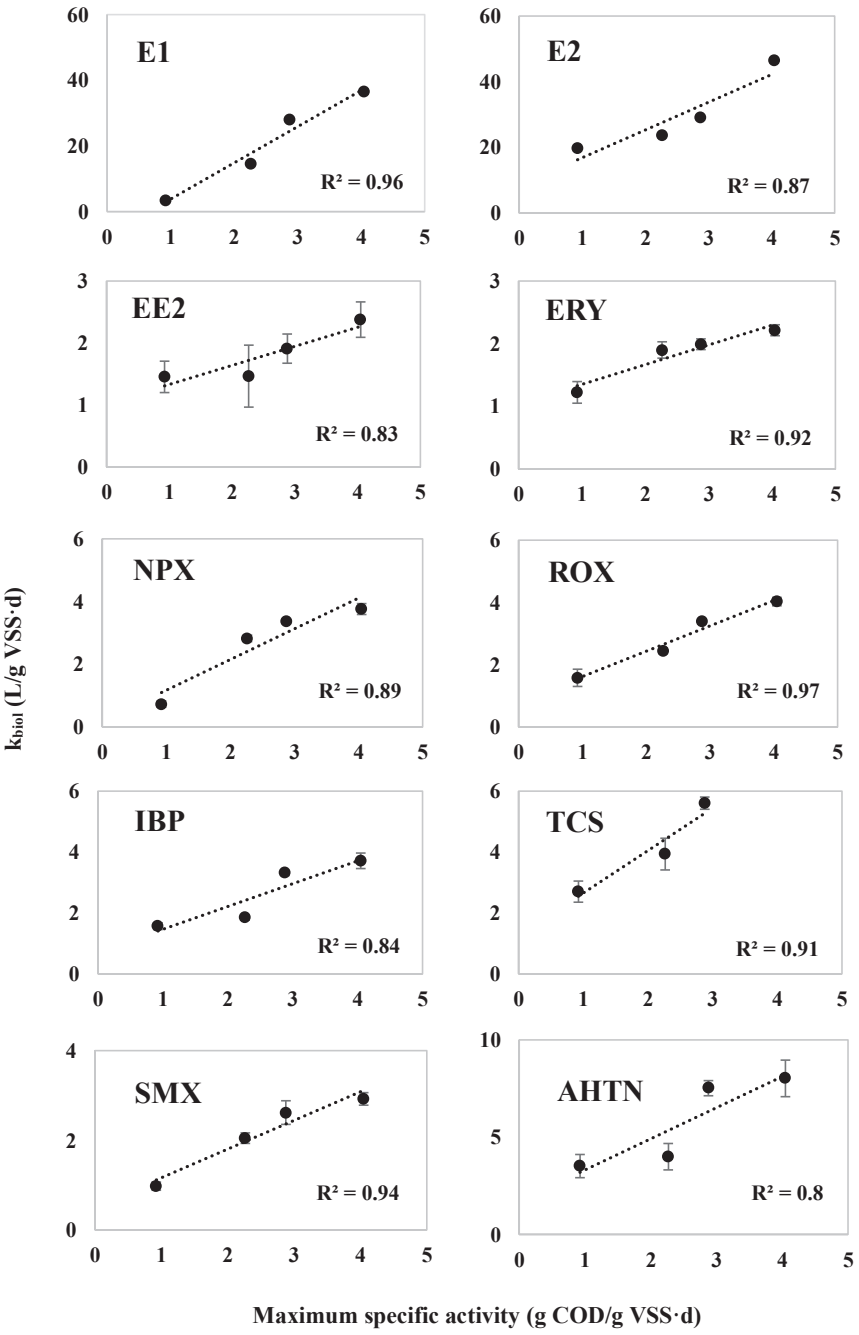


Fig. 3.3. Representation of the biotransformation trend followed by ROX, SMX, NPX and HHCB for the experimental batch with $[COD]_0$ = 0.4 g L^{-1}. Similar results were obtained for the rest of the OMPs and COD evaluated (data not shown).

As previously indicated, the k_{biol} of a particular compound depends on its physicochemical properties and the environmental conditions (pH, temperature and redox potential, among others). Interestingly, as shown in Fig. 3.4, we found out that the specific activity of the sludge, caused by the primary substrate, also affects the value of k_{biol}. A positive correlation (with an R-square value between 0.80 and 0.96) is observed between k_{biol} and the maximum COD specific activity for all non-recalcitrant OMPs, with the exceptions of HHCB and ADBI. This proves that r_{biol} is influenced by the microbial activity through the variation of k_{biol}. Since it was not seen a limitation in the increasing tendency of k_{biol}, which maintained the linearity throughout the assays, it can be speculated that higher k_{biol} values could be obtained at even higher specific COD activities. Nonetheless, we do not have enough data to prove this theory and experiments at higher heterotrophic activities would be required for confirmation.

It is worth comparing the results from Fig. 3.2 and Fig. 3.4, which showed some differences. For instance, ERY showed almost 4-fold increases in its specific biotransformation rate (Fig. 3.2) while its k_{biol} values only showed 2-fold increments (Fig. 3.4). These discrepancies were also observed in other compounds such as E1 and ROX. One explanation for such behavior could be that media conditions and especially process conditions, such as operating in batch mode rather than in a continuous manner, could lead to a different intensity in the metabolic responses, in agreement with the results from Park et al. (2018). Besides, microorganisms may have followed different enzymatic routes in the batch and reactor experiments to degrade the substrates due to variations in the initial feeding concentrations, in consonance with previous studies showing that the concentration of the metabolites plays a key role in pathway selection and the metabolic flux (Wegner et al., 2015). Despite these differences, overall both continuous and batch experiments provide consistent and complementary conclusions.

A)



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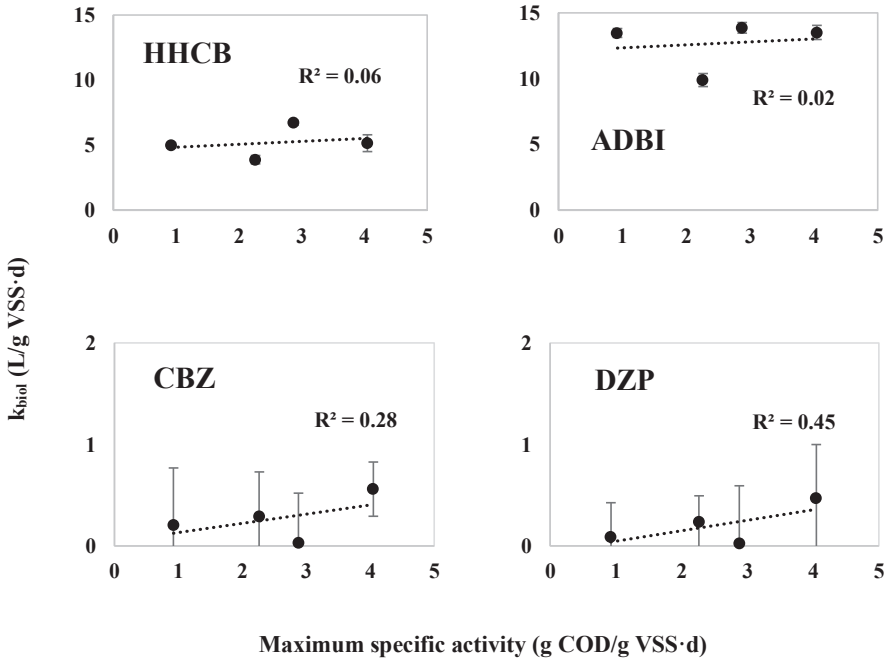

Fig. 3.4. OMPs biotransformation kinetic constant (k_{biol}) vs the maximum COD specific activity achieved in each batch assay, along with the R squared value (R^2). A) OMPs that showed a positive correlation. B) OMPs that showed a negative correlation.

Throughout the experiments, the influence of the heterotrophic activity on k_{biol} varied among the OMPs. For instance, in the case of the hormones, while E1 increased its k_{biol} almost 20-fold, E2 only increased it 2-fold. Among the fragrances, while AHTN duplicated its k_{biol}, ADBI and HHCB did not show any variation. These findings show that the influence of the heterotrophic activity is substance-specific.

The reported results show that the biotransformation of a particular OMP would depend on the characteristics of the microbial population and their specific performance. From our results, and considering the findings of Majewsky et al. (2010) and Gonzalez-Gil et al. (2018), it can be concluded that at low microbial activities, the k_{biol} of a compound does not vary, at intermediate activities the k_{biol} increases in a linear manner, and finally, when the microbial activity is sufficiently high, it reaches a plateau and k_{biol} remains constant. Our findings suggest that, for the range of heterotrophic activity studied, the majority of the selected OMPs were in the region where k_{biol} increases

linearly. The exceptions of HHCB and ADBI could indicate that they would have required lower or higher microbial activities, proving that k_{biol} is influenced both by the microbial activity and the nature of the compound.

To better understand the influence of the microbial activity on the biotransformation of the OMPs, Table 3.1 shows an estimation of the biotransformation rates that could be achieved for ROX, NPX, SMX and HHCB in a real activated sludge system designed to only remove organic matter, as well as the HRT that these OMPs would require for complete biotransformation according to the experimental k_{biol} values (Fig. 3.4). For instance, in the case of NPX, assuming an inlet concentration of 10 μg L^{-1}, a k_{biol} equivalent to 0.7 L g VSS^{-1} d^{-1} would provide a biotransformation rate of 14 μg NPX L^{-1} d^{-1} and an HRT of 17 h would be required. On the other hand, a k_{biol} of 3.8 L g VSS^{-1} d^{-1} would lead to a biotransformation rate of 76 μg NPX L^{-1} d^{-1} and an HRT of 3 h could be sufficient for total NPX removal. The results indicate that higher heterotrophic activities could considerably reduce HRT requirements thanks to improved OMPs specific biotransformation rates. Moreover, except for DZP and CBZ, which are recalcitrant under heterotrophic conditions, all the OMPs would be almost completely removed after 8 h (Table 3.1). Accordingly, promoting the heterotrophic activity with higher OLRs would not have significant effects on the biotransformation yield at typical HRTs of activated sludge systems, supporting the findings observed in Fig. 3.1.

Table 3.1. Biotransformation rate and HRT required for full biotransformation of ROX, NPX, SMX and HHCB in a real WWTP based on the k_{biol} values obtained in the batch assays. The X_{VSS} value used is a typical solids concentration in WWTPs (Metcalf & Eddy, 2014), and the C_w value is in the range of typical influent OMPs concentrations in WWTPs (Luo et al., 2014; Petrie et al., 2014).

OMPs	Specific biotr. rate (g COD g VSS^{-1} d^{-1})	k_{biol} (L g VSS^{-1} d^{-1})	X_{VSS} (g L^{-1})	C_w (μOMP L^{-1})	Biotransformation rate (μg OMP L^{-1} d^{-1})	HRT required for 100% biotransformation (h)
ROX	0.4	1.6	2	10	32	7.6
	0.6	2.4			49	4.9
	1.1	3.4			68	3.5
	2.7	4.0			81	3.0
NPX	0.4	0.7	2	10	14	17.1
	0.6	2.8			56	4.3
	1.1	3.4			68	3.5
	2.7	3.8			76	3.2
SMX	0.4	1.0	2	10	20	12.0
	0.6	2.0			40	6.0
	1.1	2.6			52	4.6
	2.7	2.9			58	4.1
HHCB	0.4	5.0	2	10	100	2.4
	0.6	3.9			78	3.1
	1.1	6.7			134	1.8
	2.7	5.2			104	2.3

3.4. CONCLUSION

In this chapter, experimental data evidenced that higher OLRs lead to higher OMPs biotransformation rates and that the removal of the organic matter and the OMPs occurs simultaneously, proving cometabolism as the main mechanism behind the biotransformation of OMPs under aerobic heterotrophic conditions. The enhancement of the OMPs biotransformation rate occurred due to increases in their respective k_{biol} values, showing that the biotransformation kinetic constant is not only dependent on the compound and the environmental conditions, but also on the intensity of the metabolic activity. Besides, the influence of the heterotrophs in k_{biol} is proved to be compound dependent. On the other hand, the cometabolic trend does not necessarily involve an improvement in the biotransformation yield of the compounds providing that a sufficient HRT is applied. In fact, in this work, it is shown that the effectiveness did not improve at higher specific degradation rates of the primary substrate. Hence, the variation of the WWTP organic load, the management of the organic substrates and the control of the microbial activity appear as key parameters governing OMPs biotransformation.

CHAPTER 4. Heterotrophic enzymatic biotransformations of organic micropollutants in activated sludge

SUMMARY

While heterotrophic microorganisms constitute the major fraction of activated sludge biomass, the role of heterotrophs in the biotransformation of organic micropollutants (OMPs) has not been fully elucidated. Yet, such knowledge is essential, particularly when conceiving novel wastewater treatment plants based on a two-stage process including an A-stage under heterotrophic conditions and a B-stage based on anammox activity. Biotransformation of OMPs in activated sludge is thought to mostly occur cometabolically thanks to the action of low specificity enzymes involved in the metabolism of the primary substrates. For a better understanding of the process, it is important to determine such enzymatic activities and the underlying mechanisms involved in OMPs biotransformation. This task has proven to be difficult due to the lack of information about the enzymatic processes and the complexity of the biological systems present in activated sludge. In this chapter, a continuous aerobic heterotrophic reactor spiked with 20 OMPs at environmental concentrations was operated to *(i)* assess the potential of heterotrophs during the cometabolic biotransformation of OMPs, *(ii)* identify biotransformation reactions catalysed by aerobic heterotrophs and *(iii)* predict possible heterotrophic enzymatic activities responsible for such biotransformations. Contradicting previous reports on the dominant role of nitrifiers in OMPs removal during activated sludge treatment, the heterotrophic population proved its capacity to biotransform the OMPs to extents equivalent to reported values in nitrifying activated sludge plants. Besides, 12 transformation products potentially formed through the activity of several enzymes present in heterotrophs, including monooxygenases, dioxygenases, hydrolases and transferases, were identified.

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Further information can be found in the “List of publications” (page 145).

4.1. INTRODUCTION

Since OMPs are frequently not fully mineralized during cometabolic biotransformation, the assessment of the TPs is also important because they pose the potential to be as or even more toxic than their parent compounds to the ecosystem (Berkner and Thierbach, 2014; Celiz et al., 2009; Gulde et al., 2016). Besides, TPs identification can be a very helpful tool to determine the reactions occurring in a specific environment and provide hints about the enzymatic activities catalysing OMPs biotransformation. For instance, following this approach, Gulde et al. (2016) demonstrated that N-acyltransferases could be involved in the biotransformation of several amine-containing OMPs in activated sludge.

In this chapter, we aim to address the contribution of heterotrophs to the biotransformation of OMPs in activated sludge processes and, particularly, their capacity to biotransform a range of OMPs. Besides, we attempt to detect several TPs as a tool to identify key OMP biotransformation reactions catalysed by aerobic heterotrophs and decipher possible enzymatic activities carrying out such biotransformations. To this end, we evaluated the biotransformation of a set of OMPs with different physicochemical properties in an aerobic heterotrophic reactor and used liquid chromatography coupled to high-resolution mass spectrometry to identify TPs.

4.2. MATERIALS AND METHODS

A 5 L continuously stirred lab-scale reactor connected to a 2 L settler (section 2.2.1.) was operated at 25ºC with an OLR of 0.6 g COD L^{-1} d^{-1}, HRT of 1 d and SRT of approximately 5 d to determine the key enzymatic activities involved in the biotransformation of several OMPs. The reactor was inoculated with activated sludge at a concentration of 1 g L^{-1} and the experimental stage was maintained for one month. The feeding included ATU to avoid the development of nitrifying activity and was based on acetate as organic carbon source (section 2.1.2.). Besides, 20 OMPs (section 2.1.1.) were spiked at concentrations of 10 µg L^{-1} for the pharmaceuticals, 1 µg L^{-1} for the hormones and 40 µg L^{-1} for the fragrances.

Once steady state was reached, inlet and outlet OMPs and TPs concentrations were measured (solid and liquid phase) by taking triplicate samples from the feeding and the reactor vessel in three consecutive days. Besides, analysis of the influent and effluent of

the reactor was performed to determine the typical operational parameters according to the standard analytical methods described in **Chapter 2**. The soluble COD, ammonium, nitrate and nitrite concentrations, TSS, SSV, pH and temperature were measured two to three times per week.

4.3. RESULTS AND DISCUSSION

4.3.1. Reactor performance

The reactor was operated for 28 days and steady-state conditions were achieved after one week. During experimentation, neutral pH was maintained to maximize the activity of the microorganisms as well as to avoid any effects of varying pH levels on OMPs removal. Although frequently receiving little attention, pH is a key parameter affecting OMPs biotransformation. Its increase by just one pH unit can considerably promote the removal of OMPs with cationic-neutral specification and hinder that of compounds with neutral-anionic speciation, as shown by Gulde et al. (2014).

Biomass concentration remained quite stable at 1.4 g L^{-1} by controlling the purge (Fig. 4.1), performed almost daily from the settler, and the control of the recirculation, set at a ratio of 100% of the influent flow (Eq. 4). The source of organic matter was acetate, which was fully consumed (95-100%), leading to effluent COD concentrations consistently below 0.03 g L^{-1} (Fig. 4.1). Besides, operation occurred in the absence of nitrification (Fig. 4.1), indicating that all the nitrogen consumed was used only for microbial growth. Among the non-consumed nitrogen, some soluble non-degradable organic nitrogen from endogenous respiration may have been present.

The net waste activated sludge produced was estimated at 0.15 g VSS L^{-1}, including the active heterotrophic biomass formed daily and the cell debris, which accounts for 10-20% of the decayed biomass and cannot be degraded due to their extremely low hydrolysis kinetics (Liu and Wang, 2015). Moreover, there was also an endogenous decay rate of biomass in the reactor, proportional to the active biomass concentration (X_{VSS}), that accounted for 0.14 g VSS L^{-1} d^{-1}. The endogenous decay represents the cell biomass loss and includes an internal decay (cell level), involving the oxidation of stored substrates to produce energy for cell maintenance, and an external decay (community level), such as cell death or predation by higher organisms (Liu and Wang, 2015). Our reactor presented mostly aerobic heterotrophic bacterial strains, as well as higher life-

forms, such as rotifers and protozoans, based on microscopic observation. The high life forms have also been shown to participate in the removal of OMPs, as reported by Gulde et al. (2018) for protozoa, which seem to be involved in the ion trapping of amine-containing compounds and the hydrolysis of select esters and phenylurea compounds.

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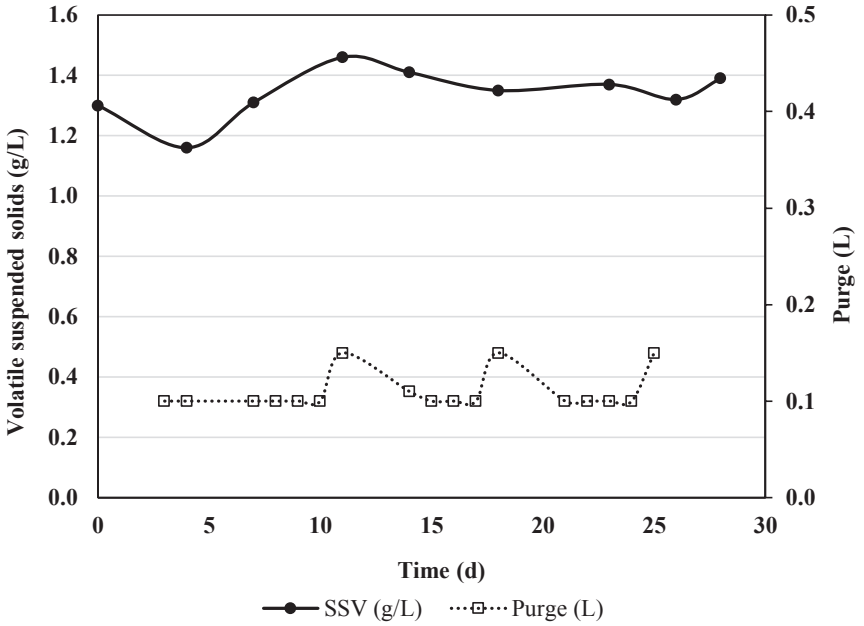

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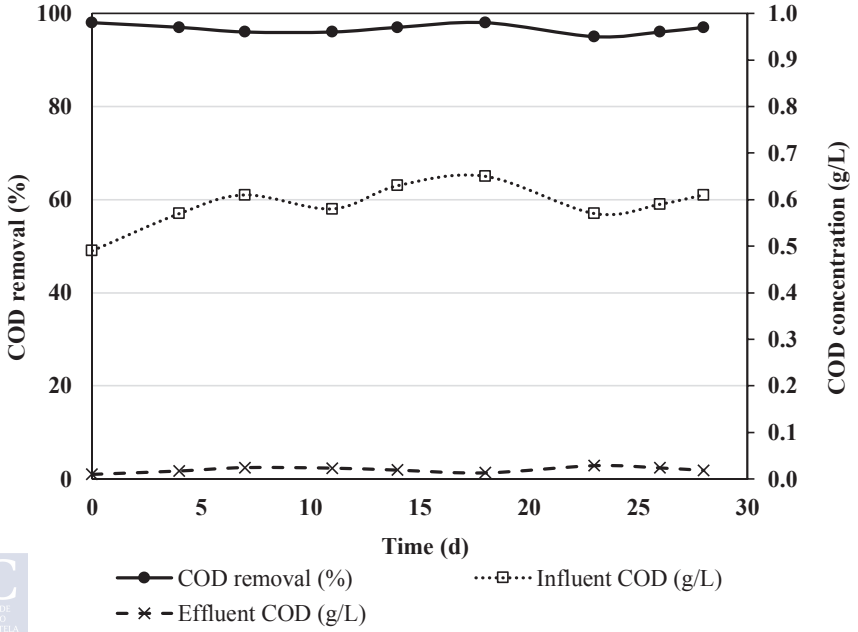

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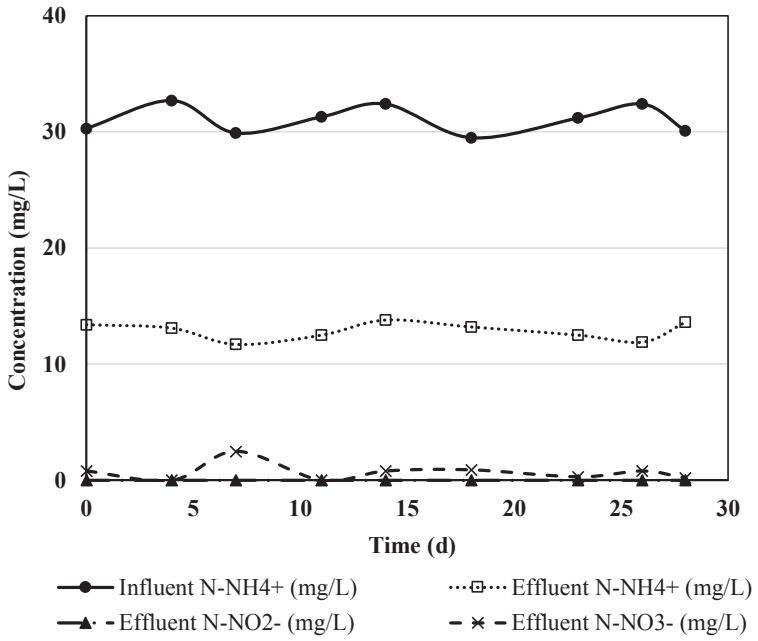


Fig. 4.1. Operational parameters obtained in the continuous aerobic heterotrophic reactor. A) VSS concentration and purge performed. B) COD removal along with COD influent and effluent concentrations. C) Influent N-NH_4^+ and effluent N-NH_4^+, N-NO_3^- and N-NO_2^- concentrations.

4.3.2. Heterotrophic OMPs biotransformation in activated sludge systems

Fig. 4.2 shows the fate of the selected OMPs in the continuous heterotrophic reactor. Sorption appears to be minimal for most compounds (<10%), except for FLX, for which it accounts for 15-20% of the total mass balance. Results show that 17 out of the 20 OMPs were highly removed (above 80%) due to heterotrophic activity and only 3 compounds were slightly (TMP) or not removed at all (CBZ, DZP). These results agree with the biotransformation observed in activated sludge units (Alvarino et al., 2014; Luo et al., 2014; Petrie et al., 2014), except for DCF, for which we obtain higher values than under typical activated sludge conditions (Alvarino et al., 2014; Fernandez-Fontaina et al., 2016). Nonetheless, in other biological treatments, high DCF efficiencies have also been achieved, as in a hybrid biofilm-activated sludge process (Jewell et al., 2016b) and in a nitrifying moving bed biofilm reactor (Torresi et al., 2016b). In our experiments, DCF

was biotransformed rather extensively, i.e., at 80% (Fig. 4.2), which could indicate the presence of certain heterotrophic genera capable of biotransforming it more efficiently. In this regard, Nguyen et al. (2019) showed that under exposure to a primary carbon substrate and DCF, certain genera of activated sludge bacteria can significantly increase their abundance, suggesting that they might gain a competitive advantage from its cometabolic removal.

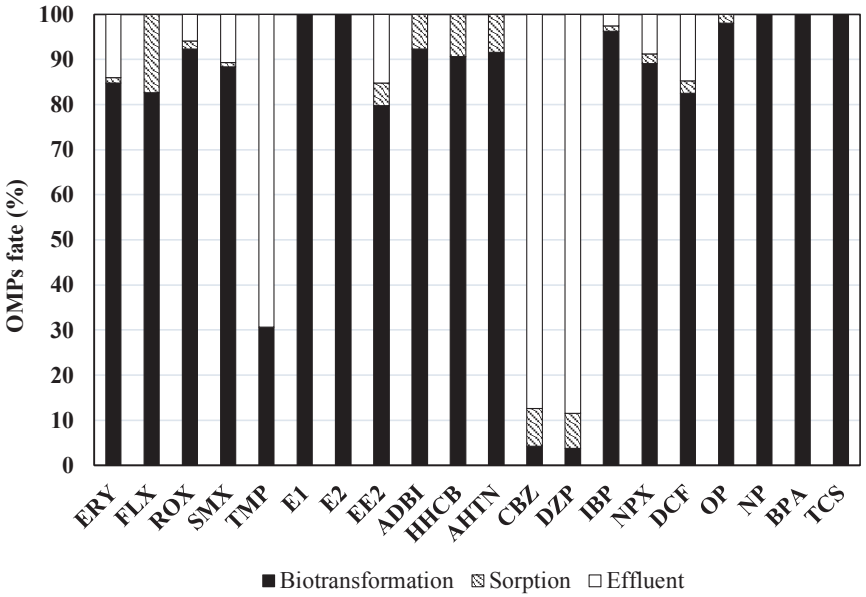


Fig. 4.2. Fate of the OMPs in the continuous aerobic heterotrophic reactor operated with fixed HRT (1 d) and OLR (0.6 g COD L^{-1} d^{-1}).

To understand the variability in OMPs biotransformation reported in the literature, Fig. 4.3 compares the biotransformation performance of our aerobic heterotrophic reactor with that reported under nitrifying conditions exclusively (systems not fed with organic carbon or that have inhibited the heterotrophic activity) and in activated sludge systems presenting both heterotrophic and nitrifying activities. Firstly, it can be observed that heterotrophs reach biotransformations similar to the values of the activated sludge plants, indicating that they can greatly contribute to the removal of the OMPs, and that, for certain compounds, there may not be the need to set up WWTPs with nitrifying activities. There is a limited number of studies evaluating the biotransformation of the selected OMPs with exclusively nitrifying populations. In such works, they have also reported

high removals for these compounds, indicating that both nitrifiers and heterotrophs have in their metabolic networks enzymes capable of biotransforming the OMPs. However, there are a few compounds that show differences in their behavior between the different conditions. While the biotransformation of SMX and TCS is higher under heterotrophic or activated sludge conditions, CBZ and DZP show better efficiencies with nitrifying populations. Moreover, although the average removal values of TMP seem similar under all conditions, it is a compound typically difficult to remove by aerobic heterotrophs (Fernandez-Fontaina et al., 2016), while some nitrifying populations have shown the capacity to biotransform it up to 50% or more (Batt et al., 2006; Fernandez-Fontaina et al., 2012). Furthermore, as discussed previously, heterotrophs display better biotransformation for DCF. Additionally, it is surprising to observe higher average biotransformations for some compounds under exclusively heterotrophic or nitrifying conditions than in activated sludge systems presenting both activities. A possible explanation could be due to differences in the process conditions under which the experiments were performed, such as applying different OLRs. However, previous studies have shown that in certain cases mixed consortia of bacteria can provide worse OMPs removals than individual populations because the composition of a mixed bacterial culture can affect their performance (Larcher and Yargeau, 2011).

Thus, based on Fig. 4.3, it can be concluded that nitrifying systems or nitrifying activated sludge systems would not be required to achieve the typical biotransformation extents of the selected OMPs since, in general, the biotransformation is similar for all microbial populations. Nonetheless, it is important to remark that the data collected from the literature was obtained from experiments performed at considerably different operational conditions, which could have affected the biotransformation extents obtained in each case and explain the high standard deviations observed for some compounds.

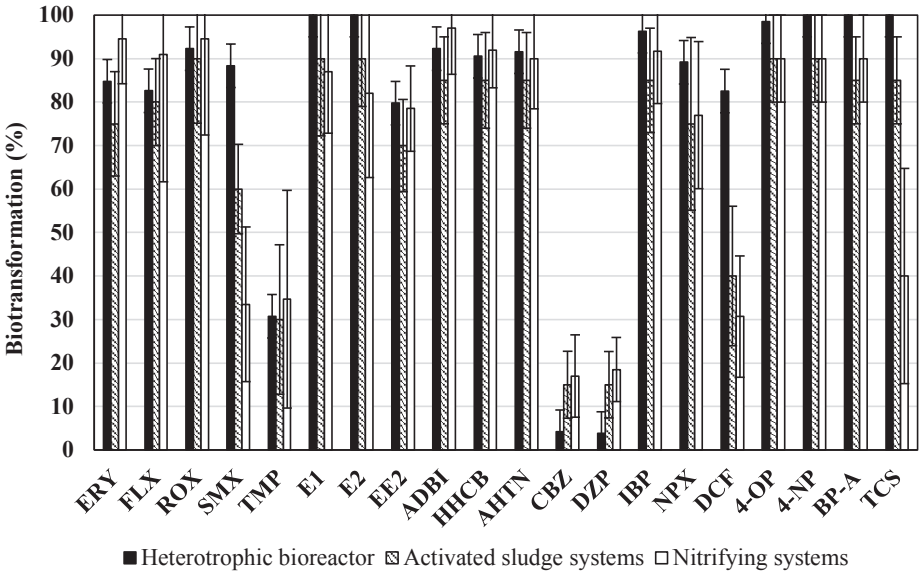


Fig. 4.3. OMPs biotransformation comparison between our heterotrophic reactor and the average literature-reported values in nitrifying systems and activated sludge systems with heterotrophic and nitrifying activities. The data was obtained from multiple studies (Alvarino et al., 2018a, 2018b; Batt et al., 2006; Fernandez-Fontaina et al., 2016, 2013, 2012; Gardner et al., 2013; Kim et al., 2007; Lee et al., 2015; Luo et al., 2014; Margot et al., 2015; Petrie et al., 2014; Suarez et al., 2010; Tran et al., 2018, 2009; Yuxin Wang et al., 2020; Yi and Harper, 2007).

4.3.3. Identification of enzymatic biotransformations of OMPs in aerobic heterotrophic conditions

In total, 12 TPs were confidently identified (Table 4.1) for the 20 investigated OMPs and assigned to three major types of transformation reactions (oxidation, hydrolysis and conjugation). For ADBI and FLX, 2 different TPs were observed, pointing towards sequences of transformation steps or transformations taking place at different functional groups. The structural confidence was considered probable (level 2) for 1 TP and tentative (level 3) for 9 TPs, whereas 2 TPs could only be assigned an unequivocal molecular formula (level 4). The confidence varied mostly depending on the availability of MS and MS2 data, literature information and the molecular structure of the TPs and respective OMPs (structural assignments to TPs containing only C, H and O atoms were generally treated with more caution).

A) Oxidation: Oxidation reactions frequently represent the initial biotransformation of xenobiotics in activated sludge systems. In our reactor, we determined several TPs that were formed through oxidative biotransformation, in the form of hydroxylation, deamination, demethylation and dehydrogenation.

A.1) Hydroxylation: Hydroxylation is commonly reported during OMPs biotransformation since it can be carried out by multiple microorganisms. For instance, AOB and AOA can hydroxylate mianserin (Men et al., 2016), the novel complete AOB (commamox), carbendazim (Han et al., 2019) and heterotrophic microorganisms, EE2 (Khunjar et al., 2011). Our results show heterotrophic hydroxylation of ADBI, HHCB, E1, E2, DCF and NP (Table 4.1), likely catalyzed by low specificity monooxygenases, such as cytochrome P450 or flavin-containing monooxygenases, or dioxygenases, such as Rieske-type non-heme-iron dioxygenases (Bjørseth and Angeletti, 1986). The most common biotransformation reaction of steroid hormones, including E1 and E2, is hydroxylation, performed by oxygenases in multiple organisms, such as bacteria, fungi or algae (Pratush et al., 2020). Fragrances can also undergo hydroxylation, as reported for HHCB, in bacteria, algae and fungi, where the initial biotransformation reaction is hydroxylation at different carbon positions (Ding et al., 2020; Martin et al., 2007). In the case of DCF, there is limited information about its biotransformation reactions in WWTPs. However, hydroxylation has been observed as the first reaction in its biotransformation pathway in activated sludge plants, being considered a bottleneck due to its low rate, which could explain the frequently limited removal yield (Bouju et al., 2016). In our reactor, DCF was highly biotransformed (Fig. 4.2) and the corresponding hydroxylated TP was found, possibly indicating that heterotrophs were able to perform the hydroxylation step efficiently. Differently from DCF, NP hydroxylation in WWTPs has not been reported before, to the best of our knowledge. Nonetheless, in mammals and fish, hydroxylation both in the ring and the alky chain has been observed, possibly due to the action of cytochrome P450 enzymes (Thibaut et al., 2002).

A.2) Deamination: A TP detected for FLX (Table 4.1) was either *(i)* the product of deamination at the secondary amine group followed by oxidation of the resulting aldehyde or *(ii)* the outcome of initial demethylation, followed by deamination of the resulting primary amine and a final oxidation of the formed aldehyde. The second pathway seems more plausible since secondary amines typically undergo dealkylation before oxidative deamination. Nonetheless, in certain cases, direct deamination of

secondary amines may occur, as in the metabolism of propranolol, where the biotransformation can occur through the desisopropyl primary amine metabolite or by a direct oxidative deamination reaction yielding an aldehyde metabolite and isopropylamine (Beale and Block, 2011). Gulde et al. (2016) observed deaminations for some primary, secondary and tertiary OMPs, such as primaquine, N-demethylvenlafaxine and pyrilamine, although they did not observe FLX deamination.

Assuming the previous demethylation, the deamination step in FLX could be performed by an amine oxidase, catalyzing the oxidative cleavage of the primary amine to an aldehyde while releasing ammonia and hydrogen peroxide. These enzymes are found in multiple organisms controlling the level of amines, participating in multiple pathways, and allowing various amine substrates to be used as sources of carbon and nitrogen in prokaryotes (Messerschmidt, 2010). The following oxidation of the aldehyde to the carboxylic acid is likely catalyzed by aldehyde dehydrogenase (using oxygen from a water molecule and NAD^+ or $NADP^+$ as cofactors), which is present in a broad range of anabolic and catabolic pathways and key in the elimination of endogenous and exogenous toxic aldehydes (Riveros-Rosas et al., 2019).

The hypothetical N-demethylation of the secondary amine of FLX would form seproxetine as a TP, the most important FLX active metabolite observed in humans and WWTPs. In human metabolism, the biotransformation occurs thanks to cytochrome P450 (Von Moltke et al., 1997), so likely a monooxygenase could be the responsible enzyme in the heterotrophic reactor. The conversion of FLX to seproxetine in activated sludge is very relevant since there is an enantioselectivity preference for (S)-FLX biotransformation. This event leads to (S)-seproxetine formation and (R)-FLX accumulation (unless enantiomerization processes take place), which for some microorganisms can be 10 and 30 times more toxic than (S)-FLX, respectively, and cause an overall increase in toxicity (Andrés-Costa et al., 2017).

A.3) Demethylation: Demethylations are catalyzed by demethylases from a variety of enzyme families, including monooxygenases (Robb et al., 2018) and dioxygenases (Fedeles et al., 2015). N-demethylation is the most common reaction and is performed by oxidative demethylases exploiting the weak C-H bonds adjacent to amines and acting on the N-methyl groups. During the process, an oxygen atom is inserted into the C-H bond and then a spontaneous decomposition of the hydroxylated intermediate occurs, forming formaldehyde and a demethylated product. O-demethylation reactions can also happen,

particularly during ether cleavage. N-demethylation of OMPs has been reported for several amines, such as pargyline, and O-demethylation has been observed in anaerobic conditions for venlafaxine (Falås et al., 2016) and in activated sludge for TMP, likely due to the action of monooxygenases (Jewell et al., 2016a; Krah et al., 2016).

Besides the possible previously mentioned demethylation of FLX to seproxetine, a N-demethylation of the tertiary amine of DZP was detected (Table 4.1), leading to the formation of nordazepam, a very active metabolite with a long half-life that has been found in humans (Kosjek et al., 2012). In human metabolism, the enzyme carrying out the biotransformation is a cytochrome P450 (Luk et al., 2014), thus, it is likely that in our bioreactor a monooxygenase was also the responsible enzyme.

A.4) Dehydrogenation: Besides hydroxylation, further oxidations were seen for ADBI and NP (Table 4.1). They could have been catalyzed by alcohol dehydrogenases or oxidases (depending on the electron acceptor), which are capable of forming the carboxylic acid moiety (Phale et al., 2019). These enzymes are present in many organisms, including bacteria, and play a crucial role in many metabolic pathways, as in the reversible reaction where acetaldehyde is converted to ethanol by alcohol dehydrogenase, a key step to regenerate the cofactors required for glycolysis.

B) Hydrolysis: Hydrolysis reactions are carried out by hydrolases, which have been previously observed in activated sludge, including during the biotransformation of atenolol and azoxystrobin by bacterial amidohydrolases and protozoan hydrolases, respectively (Achermann et al., 2018b). The reported hydrolases in literature belong to several catalytic classes, including amidases, esterases, phosphatases and peptidases, among others (Krah et al., 2016). Here, we report for TMP a hydrolytic displacement of a primary amine moiety (Table 4.1), a novelty compared to reported TMP biotransformations, involving mostly demethylation and oxidation reactions (Jewell et al., 2016a). It is hypothesized that the TP formed through hydrolysis of the aminopyrimidine group of TMP (Table 4.1) could be obtained thanks to aminopyrimidine aminohydrolase (EC 3.5.99.-), involved in the metabolism of thiamine, a vitamin required by organisms from many life kingdoms, including bacteria (Lemmer and Nitschke, 1994). Hydrolysis of amine moieties in OMPs is rather rare since they more often undergo N-oxidation, α-C-hydroxylation and conjugation reactions (Gulde et al., 2016). However, this type of biotransformation has been observed before, as in reactions involving chorismic acid (Ganem, 1995), a metabolite present in the shikimic acid pathway,

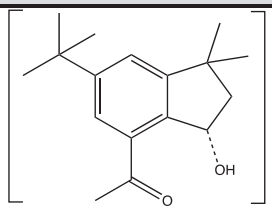
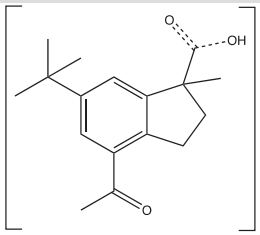
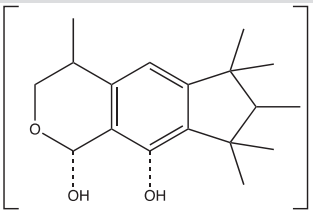
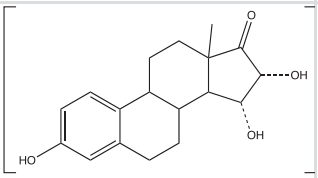
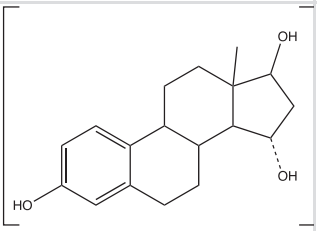
followed by bacteria and other organisms for the biosynthesis of folates and aromatic amino acids.

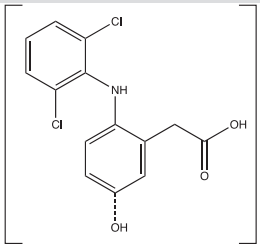
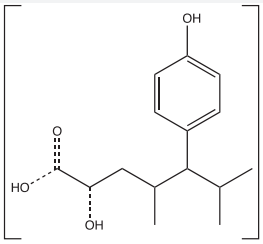
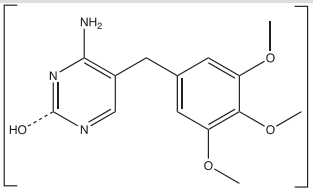
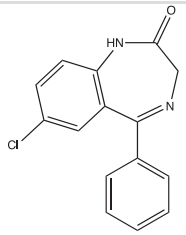
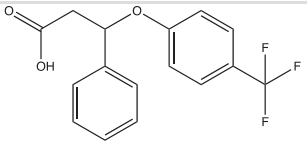
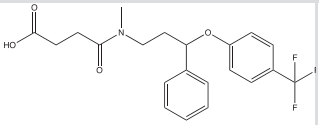
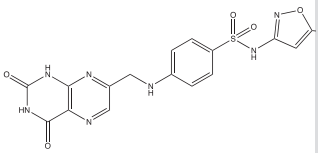
C) Conjugation: Conjugation reactions are anabolic and catalyzed by transferases thanks to the presence of a functional group in the substrate that serves as the anchoring site for a molecule or moiety. Since these types of reactions can occur both with exogenous and endogenous substrates, they play a key role in the metabolism of xenobiotics (Testa and Krämer, 2008).

We observed a conjugation reaction at the amine group of SMX (Table 1), leading to a TP found by Achermann et al. (2018a) in the effluent of several WWTPs. The biotransformation pathway consists of a pterin conjugation, an oxidation step and a final hydrolysis reaction. The enzymes involved in the process could belong to the folic acid pathway, such as pterin deaminase and dihydropteroate synthase. If the folic acid synthesis is the responsible pathway, the biotransformation would be linked to bacterial growth and maintenance, which could explain the generally good biotransformation observed in various environments for SMX, including anaerobic, nitrifying and heterotrophic conditions (Achermann et al., 2018a).

Besides, a FLX TP was formed by conjugation at the amine moiety (Table 4.1) following an N-acylation reaction (N-succinylation), suggesting N-acyltransferases as the responsible enzymes (Gulde et al., 2016). N-acylation reactions are important in microbial xenobiotic metabolism, with N-succinylation being particularly involved in the biotransformation of primary and secondary amines to secondary and tertiary amines, respectively.

Table 4.1. Summary of the suggested TPs structures found in the aerobic heterotrophic reactor, including the confidence level, atom changes relative to the parent OMPs and biotransformation reactions and enzymes likely involved in the process. The TP structures placed in brackets are only suggestions for possible structures. The functional groups bonded with dotted lines could be placed somewhere else in the molecule.

OMPs	TP	Level	Atom change	Reaction	Candidate enzymes
ADBI		3	+ O	Hydroxylation	EC 1.14.- EC 1.13.-
ADBI		3	+ 2O - 2H	Hydroxylation Additional oxidation	EC 1.14.- EC 1.13.- EC 1.1.-
HHCB		4	+ 2O	Hydroxylation	EC 1.14.- EC 1.13.-
E1		4	+ 2O	Hydroxylation	EC 1.14.- EC 1.13.-
E2		3	+ O	Hydroxylation	EC 1.14.- EC 1.13.-

DCF		3	+ O	Hydroxylation	EC 1.14.- EC 1.13.-
NP		3	+ 3O - 2H	Hydroxylation Additional oxidation	EC 1.14.- EC 1.13.- EC 1.1.-
TMP		3	+ O - N, H	Hydrolysis	EC 3.5.99.-
DZP		3	- C, 2H	Demethylation	EC 1.14.-
FLX		3	+ 2O - C, N, 5H	Demethylation Deamination Oxidation	EC 1.14.- EC 1.4.- EC 1.2.1.-
FLX		2	+ 4C, 3O, 4H	Conjugation	EC 2.3.-
SMX		3	+ 7C, 4N, 2O, 4H	Conjugation	EC 2.5.- EC 3.5.4.11

4.4. CONCLUSION

This chapter highlights the relevant role of aerobic heterotrophs in the cometabolic biotransformation of OMPs, speaking to their capacity to contribute to the overall removal of OMPs in activated sludge plants and questioning the requirement of maintaining a nitrifying activity. In fact, this study shows that heterotrophs can achieve similar extents of biotransformation for the selected OMPs as those reported in several literature studies with activated sludge (with nitrifying, denitrifying and heterotrophic activities) and purely nitrifying systems. Based on the TPs produced, it was possible to identify the main reactions involved in OMPs biotransformation under aerobic heterotrophic conditions, which are oxidation (hydroxylation, dehydrogenation, deamination and demethylation), hydrolysis and conjugation routes. An overall analysis of all results allows selecting mono- and dioxygenases, dehydrogenases, hydrolases and transferases as some of the main enzymatic activities likely responsible for OMPs biotransformation under heterotrophic conditions. Thus, this chapter highlights the relevant contribution of heterotrophs to OMPs removal, which are gaining importance in the conception of new WWTPs, and deepens the knowledge on their biotransformation mechanisms.

CHAPTER 5. Sulfamethoxazole triggers specific enzymatic activities under aerobic heterotrophic conditions: A metaproteomic approach

SUMMARY

Wastewater treatment plants (WWTPs) are a potential hotspot for the development of antibiotic resistance genes, creating the need to study the fate of antibiotics and the evolution of the microbiota when exposed to these compounds. This chapter aims at determining whether the presence of sulfamethoxazole (SMX), even at low concentrations, modifies the microbial structure and enzymatic expression of a heterotrophic activated sludge system. To that end, a combined approach including metaproteomic, genomic and transformation product analyses was followed. Results revealed the formation of the metabolites N^4-acetyl-SMX and 2,4(1H,3H)-pteridinedione-SMX, pointing out the presence of several biotransformation pathways. Besides, when exposed to increasing SMX concentrations, five bacterial families belonging to the phylum Proteobacteria followed characteristic trends, the genus *Corynebacterium* increased its abundance and five enzymes involved in its central metabolism showed a differential expression, suggesting their relevance to mitigate SMX risks. Overall, this chapter confirms the potential of metaproteomic techniques to unravel organic micropollutants' biotransformation mechanisms in WWTPs and elucidate the bacterial and enzymatic key players.

This chapter was redrafted after the following under-review publication:

Kennes-Veiga, D. M., Trueba-Santiso, A., Gallardo-Garay, V., Lema, J. M., Carballa, M. Sulfamethoxazole triggers specific enzymatic activities under aerobic heterotrophic conditions: A metaproteomic approach. Environmental Science and Technology. ISSN: 1520-5851. *Under review*.

Further information can be found in the "List of publications" (page 145).

5.1. INTRODUCTION

The presence of antibiotics in WWTPs poses a serious environmental and health risk due to the development of antibiotic-resistant genes and bacteria (Pazda et al., 2019; Wang et al., 2021; World Health Organization, 2021). Sulfonamides are a group of antibiotics particularly important due to their intensive utilization worldwide and, among them, SMX is the most broadly consumed one (Achermann et al., 2018a; Carvalho and Santos, 2016). In 2020, due to the ongoing growing concern, SMX was included in the "Surface Water Watch List" published by the Water Framework Directive of the European Union to monitor and gather data about its potential risks to the aquatic environment (European Commission, 2020).

The knowledge of the enzymes and microorganisms involved in SMX biotransformation in activated sludge systems is still very limited. However, metaproteomics offers a suitable approach to solve these questions since it provides a global view of the proteins expressed by a microbial community at a specific moment (Rodríguez-Valera, 2004), allowing to study biological processes in their native environment while avoiding the time-consuming labor of isolating microorganisms (Lacerda and Reardon, 2009; Yuqiu Wang et al., 2020).

The goal of this chapter was to obtain a better insight into the underlying mechanisms involved in the aerobic heterotrophic biotransformation of SMX. Specifically, it is explored how SMX concentrations affect the biotransformation capacity, taxonomic composition, and enzymatic expression of a heterotrophic activated sludge microbial community. For that purpose, a combination of metaproteomic, genomic and TPs analyses was applied.

5.2. MATERIALS AND METHODS

A 2 L continuously stirred lab-scale reactor, connected to a 1 L settler (section 2.2.1.), was operated in three different stages. Firstly, the bioreactor was fed without SMX for two months (control stage), then with 50 µg L^{-1} of SMX for 45 days (stage I) and finally with 1000 µg L^{-1} of SMX for another 45 days (stage II). The feeding included ATU to avoid the development of nitrifying activity, was based on acetate as organic carbon source (section 2.1.2.) and the reactor was operated with an OLR of 0.5 g COD L^{-1} d^{-1}, an HRT of 1 d and a SRT of approximately 5 d. Once steady state was reached at each

stage, inlet and outlet SMX concentrations (section 2.3.5.) were measured by taking three samples from the feeding and the reactor vessel in three consecutive days. Besides, at the end of each stage, triplicate samples for TPs and 16S rRNA gene sequencing analyses were taken from the reactor (sections 2.3.6. and 2.3.7.). Furthermore, analyses of the influent and effluent of the reactor were performed to determine the typical operational parameters according to the standard analytical methods described in **Chapter 2**. The soluble COD, ammonium, nitrate and nitrite concentrations, SSV, pH and temperature were measured two to three times per week.

Additionally, a total of 18 sequential batch reactors (section 2.2.2.) divided into six groups of triplicates were spiked with SMX to obtain the following concentrations: 0 (control), 50, 250, 500, 1000 and 2000 μg L^{-1}. The reactors were inoculated with activated sludge at a concentration of 1 g L^{-1}, operated at 25ºC and the feeding was also based on acetate as organic carbon source (section 2.1.2.). The experiment lasted 25 d and was performed under sterile conditions. The content of the reactors was centrifuged daily to separate the biomass from the supernatant, which was removed before adding new feed together with the SMX spike. The operation was monitored by measuring the typical parameters according to the standard analytical methods described in **Chapter 2**. TSS, VSS and ammonium, nitrate, nitrite and oxygen concentrations were determined weekly, while the acetate concentration and pH, daily. Besides, SMX and TPs concentrations were determined on days 2, 17 and 25. Finally, samples for proteomic (days 1 and 25, section 2.3.8.) and 16S rRNA gene sequencing (day 25) analyses were taken to determine the key enzymatic and microbial activities involved in the biotransformation of SMX.

5.3. RESULTS AND DISCUSSION

5.3.1. SMX biotransformation under aerobic heterotrophic conditions

In the continuous bioreactor, the presence of SMX did not affect the consumption of the primary carbon source (acetate), possibly due to one of the following reasons: *i)* the highest SMX concentrations evaluated are still below inhibitory levels and *ii)* the role of specific bacteria involved in the biotransformation of SMX mitigated the potential negative effects of the antibiotic over other microorganisms. The biotransformation efficiency of SMX was independent of its concentration in the feeding, being just slightly lower in stage I (70 %) than in stage II (80 %). As a consequence, the specific

biotransformation rate increased considerably between the two stages, varying from 0.8 to 10.6 μg OMP g^{-1} VSS h^{-1}.

Similarly, in the sequential batch reactors, even the highest SMX concentrations did not affect the biodegradation of acetate, whose removal remained constant at 140 mg L^{-1} h^{-1}. The average SMX biotransformation ranged between 62 and 78 % depending on the spiked concentration (Table 5.1) and showed clear characteristic trends. Firstly, regardless of the SMX influent concentrations, the biotransformation yield increased from day 2 to day 17 and decreased on day 25 to values even lower than those observed on day 2. Secondly, on days 2 and 25, lower initial SMX concentrations lead to higher biotransformation yields, while this trend was not observed on day 17. The improved biotransformation on day 17 compared to day 2, as well as the reduced biotransformation yield of SMX on day 2 at higher initial concentrations can be attributed to the acclimation phase of the microorganisms capable of biotransforming SMX. Their higher abundance on day 17 might have allowed reaching a constant biotransformation extent in the 80-90 % range in all bioreactors. This agrees with the findings of Li et al. (2016), who after an extended lag phase proportional to the doses amended, observed biotransformation of multiple antibiotics by bacteria from different genera. Nonetheless, the decreased biotransformation on day 25, intensified at higher SMX concentrations, might be associated with the accumulation of TPs that limit the biotransformation of the parent compound due to reversibility events (Gonzalez-Gil et al., 2019a), thermodynamic limitations (Gonzalez-Gil et al., 2018a) or by exerting toxicity over a certain concentration (Cao et al., 2019; Pérez et al., 2005), thus outweighing the increased presence of SMX degraders.

These results highlight the capacity of the aerobic heterotrophic population to extensively biotransform SMX, following the results of previous works (Fernandez-Fontaina et al., 2016; Kennes-Veiga et al., 2020; Majewsky et al., 2011). In fact, heterotrophs could be more favorable to biotransform SMX than nitrifying microorganisms (Fernandez-Fontaina et al., 2016; Tran et al., 2013), suggesting that stimulating the heterotrophic activity in activated sludge systems may positively influence its biotransformation.

Table 5.1. SMX biotransformation yield in the sequential batch reactors throughout the experiment.

Influent SMX concentration (µg/L)	Biotransformation (%)			
	Day 2	Day 17	Day 25	Average
50	80	86	68	78 ± 8
250	77	87	63	76 ± 10
500	72	79	60	70 ± 8
1000	70	79	36	62 ± 19
2000	63	86	43	64 ± 18

5.3.2. TPs identification under aerobic heterotrophic conditions

Two SMX TPs were detected in the continuous bioreactor: 2,4(1H,3H)-pteridinedione-SMX (PtO-SMX) and N^4-acetyl-SMX. The latter was only observed during stage II, which can be attributed to two reasons: *(i)* N^4-acetyl-SMX may have been also present during stage I, but at a concentration below the limit of identification, or *(ii)* additional biotransformation routes may have appeared at higher SMX doses due to variations in the concentrations of the metabolites that affected the metabolic flux and pathway selection (Wegner et al., 2015). Actually, OMPs influent concentration is recognized as a key parameter affecting biotransformation yields, rates and pathways (Onesios-Barry et al., 2014; Rios-Miguel et al., 2021; Yuxin Wang et al., 2020). For instance, Jewell et al. (2016) observed variations in the biotransformation pathway and TPs formation of TMP depending on the initial spiked concentration.

Differently, in the sequential batch reactors, only PtO-SMX was detected. It was observed in all reactors spiked with SMX at all sampling points, except for the samples taken on day 2 from the reactor fed with 50 µg L^{-1} of SMX, which is attributed to its expected low concentration in the sample.

Multiple SMX TPs formed through a wide range of reactions such as hydroxylation, acetylation, deamination or nitration have been previously reported (Majewsky et al., 2014). PtO-SMX has been detected before in WWTPs effluents and lab experiments using activated sludge (Achermann et al., 2018a). Its formation occurs through the pterin-conjugation pathway when sulfonamides interact with dihydropteroate synthetase, hindering folic acid synthesis through competitive inhibition. The pathway begins with SMX conjugation, is followed by oxidation to pterin-SMX and continues with a

hydrolysis step to form PtO-SMX thanks to the action of pterin deaminase (EC 3.5.4.11). PtO-SMX can be further broken down through various steps possibly involving oxidation and decarboxylation reactions (Achermann et al., 2018a). Most TPs formed in the sulfonamide pterin-conjugation pathway are modified at the *para* amino group and still possess the sulfanilamide toxicophore, requiring a detailed risk assessment due to their antibiotic activity, capacity to promote antibiotic resistance and ability to be back-transformed to SMX (Majewsky et al., 2015, 2014; Yun et al., 2012).

N^4-acetyl-SMX is formed through N-acylation of SMX, likely catalyzed by N-arylamine acetyltransferase (EC 2.3.1.5) (Fischer and Majewsky, 2014; X. Li et al., 2016; Tang et al., 2018). It represents the main SMX human metabolite and has been often detected in WWTPs effluents (Achermann et al., 2018a; Majewsky et al., 2014; L. J. Zhou et al., 2019). Besides, it possesses antibacterial activity and exerts toxicity, although at reduced levels compared to SMX (Majewsky et al., 2014), and it can be rapidly backtransformed to the parent compound (Achermann et al., 2018a; Radke et al., 2009), hindering the accurate assessment of its environmental relevance.

5.3.3. Impact of SMX on microbial community structure

The results obtained in the continuous bioreactor by DNA metabarcoding at the family level show a clear shift in the bacterial community related to the presence of SMX and its initial concentration in the feeding (Fig. 5.1.). This finding agrees with previous studies pointing out that the microbial structure can change under the selection pressure of many OMPs (Aguilar-Romero et al., 2020; Vasiliadou et al., 2018; G. Zhou et al., 2019). However, as previously mentioned and different from other studies (Jiang et al., 2017), the consumption of the main carbon source was not affected by these changes. Additionally, the variations in the microbial community between stages did not translate into changes in the SMX removal efficiency, which was maintained in the 70-80 % range.

Overall, the highlight is the characteristic trends followed by five bacterial families (*Rhodobacteraceae*, *Comamonadaceae*, *Xanthobacteraceae*, *Devosiaceae* and *Hyphomicrobiaceae*) belonging to the phylum *Proteobacteria*. Such phylum has been described before as one of the most dominant and prevalent in aerobic activated sludges and it has been linked to OMPs removal (Bains et al., 2019; Balcom et al., 2016; Gallardo-Altamirano et al., 2019; Vasiliadou et al., 2018).

Commamodaceae was the only bacterial family negatively affected by the presence of SMX at all stages, varying its relative abundance from 17.5 % in the control stage to

3.9 and 1.1 % during stages I and II, respectively. Interestingly, *Commamodaceae* is the only *Betaproteobacteria* of the 5 families, since the others belong to the *Alphaproteobacteria* class, which has been shown to thrive in activated sludge systems in presence of several OMPs (Davids et al., 2017).

Hyphomicrobiaceae was positively affected when SMX was added to the reactor. It was not detected in the control stage but then its abundance increased to 0.6 and 4.0 % with SMX during stages I and II, respectively. In previous studies, its abundance was related to improved OMPs removal (Coll et al., 2020; Zhang et al., 2020), although in this work it was not observed a variation in the removal efficiency of SMX between stages.

Devosiaceae showed a fast and stable adaptation to SMX. It was positively affected by the presence of SMX at 50 µg L^{-1}, increasing its abundance from 0.6 % in the control stage to 10.6 %, which then slightly dropped when SMX was spiked at 1000 µg L^{-1}.

The sum of the relative abundances of *Rhodobacteraceae* and *Xanthobacteraceae* remained approximately constant throughout the three experimental stages, suggesting a balance between them whereby when one increased, the other decreased, and vice versa. *Rhodobacteraceae*, which was initially highly abundant (70 %) and has previously been linked to improved removal efficiencies of several OMPs (Gallardo-Altamirano et al., 2019), showed a very particular trend. When SMX was spiked at 50 µg L^{-1}, its abundance decreased approximately by half, but then, when the concentration was changed to 1000 µg L^{-1}, the relative abundance reached values close to those of the control stage. It is hypothesized that firstly it was outcompeted by other microorganisms that had a faster adaptation to the introduction of SMX. Nonetheless, the capacity of *Rhodobacteraceae* to withstand the highest concentrations of SMX may have allowed them to increase their abundance again.

Conversely, *Xanthobacteraceae* was positively affected by the presence of SMX at 50 µg L^{-1} but its relative abundance dropped when SMX was spiked at 1000 µg L^{-1}. This suggests that it thrived when the SMX concentration was low but that it could have been outcompeted when SMX was added at much higher concentrations. In this sense, it has been reported before that lower OMPs concentrations may improve microbial richness and diversity (Jiang et al., 2017; Vasiliadou et al., 2018; Zhang et al., 2020; G. Zhou et al., 2019). However, higher concentrations may cause the opposite, as observed in hospital WWTPs, which typically have high pharmaceutical concentrations and reduced bacterial diversity in comparison to urban wastewaters (Vasiliadou et al., 2018).

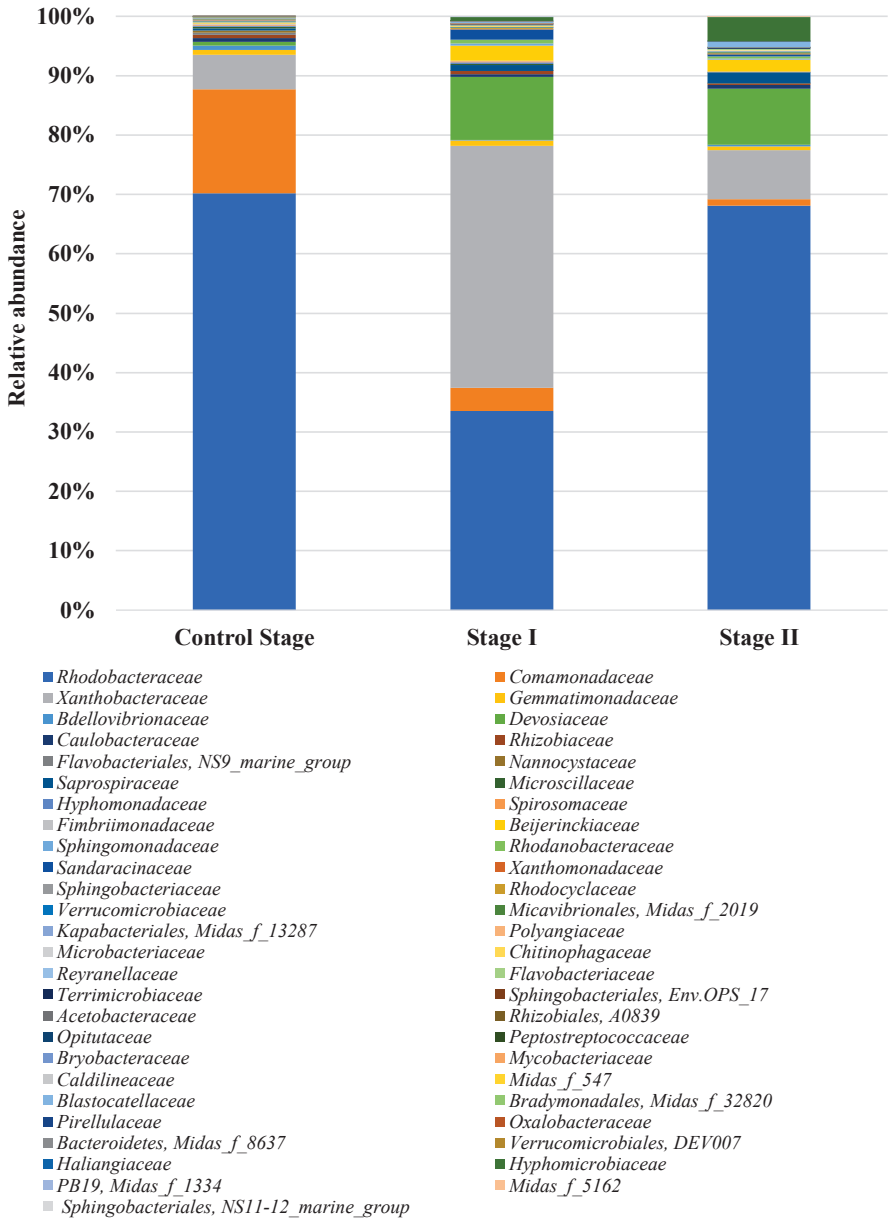


Fig. 5.1. Taxonomic composition of the microbial community at the family level in the continuous bioreactor treating SMX during the control stage (0 µg L^{-1}), stage I (50 µg L^{-1}) and stage II (1000 µg L^{-1}) according to DNA metabarcoding.

In the sequential batch reactors, the results obtained by DNA metabarcoding also showed a clear shift in the bacterial community related to the presence of SMX (Fig. 5.2).

Firstly, the abundance of *Actinobacteriota* increased proportionally to the SMX concentration. Among the genera of this phylum, *Corynebacterium* was predominant (> 75 % abundance) under all SMX concentrations. Secondly, the addition of SMX negatively affected *Campylobacterota*, which reduced its relative abundance from 67 % in the absence of SMX to 20 % at the highest SMX concentration. Finally, *Proteobacteria* and *Firmicutes* were not significantly affected by SMX, while *Bacteroidota* and *Actinobacteriota* increased their abundance.

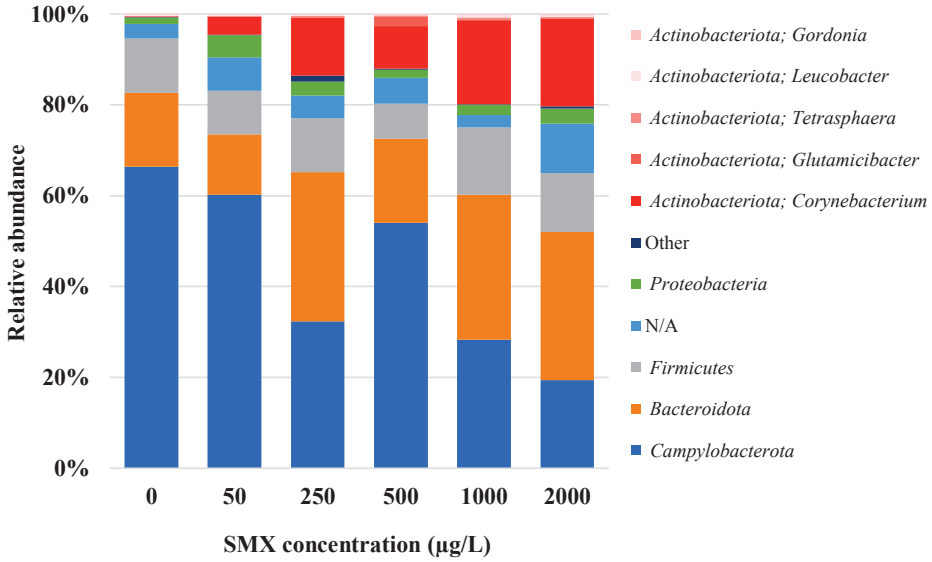


Fig. 5.2. Taxonomic composition of the microbial community on day 25 of operation of the sequential batch reactors according to DNA metabarcoding. Results are shown at the phylum level, except for the genera belonging to *Actinobacteriota*, which are displayed individually. Phyla represented ≤1 % abundance are clustered as "Other", while "N/A" refers to the population that was not taxonomically assigned.

5.3.4. Impact of SMX on the metaproteome

A total of 1051 proteins from 114 bacterial genera were identified in the analysis of the proteome samples collected on day 25 of operation of the sequential batch reactors. The proteins identified were mostly related to cell maintenance, translation, ATPase activity and the tricarboxylic acid (TCA) cycle. None of the enzymes previously linked in the literature to SMX biotransformation, including those involved in the SMX pterin-conjugation pathway and, particularly, pterin deaminase, which catalyzes the

biotransformation of Pterin-SMX to PtO-SMX, were detected in this study. This event can be attributed to their expected low relative abundance in comparison to housekeeping-related proteins or those involved in central carbon metabolism.

Fig. 5.3. represents the contribution of each bacterial genus in the inoculum and the sequential batch reactors on day 25 based on the metaproteomic results. Overall, the proteins of *Burkholderia* and *Acinetobacter* dominated the proteomes in the reactor samples, accounting jointly for 32.6 % to 43.4 %. A marked increase in the abundance of *Acinetobacter* on day 25 compared to the inoculum was observed in all reactors, including those without SMX addition, which is attributed to the operational conditions and the consumption of acetate. Interestingly, there was an increase in the abundance of *Corynebacterium* with the SMX concentration up to 500 μg L^{-1}, remaining then constant (Fig. 5.4). Therefore, both genomic (Fig. 5.2) and metaproteomic (Fig. 5.4) results point out *Corynebacterium* strains as key players in SMX biotransformation.

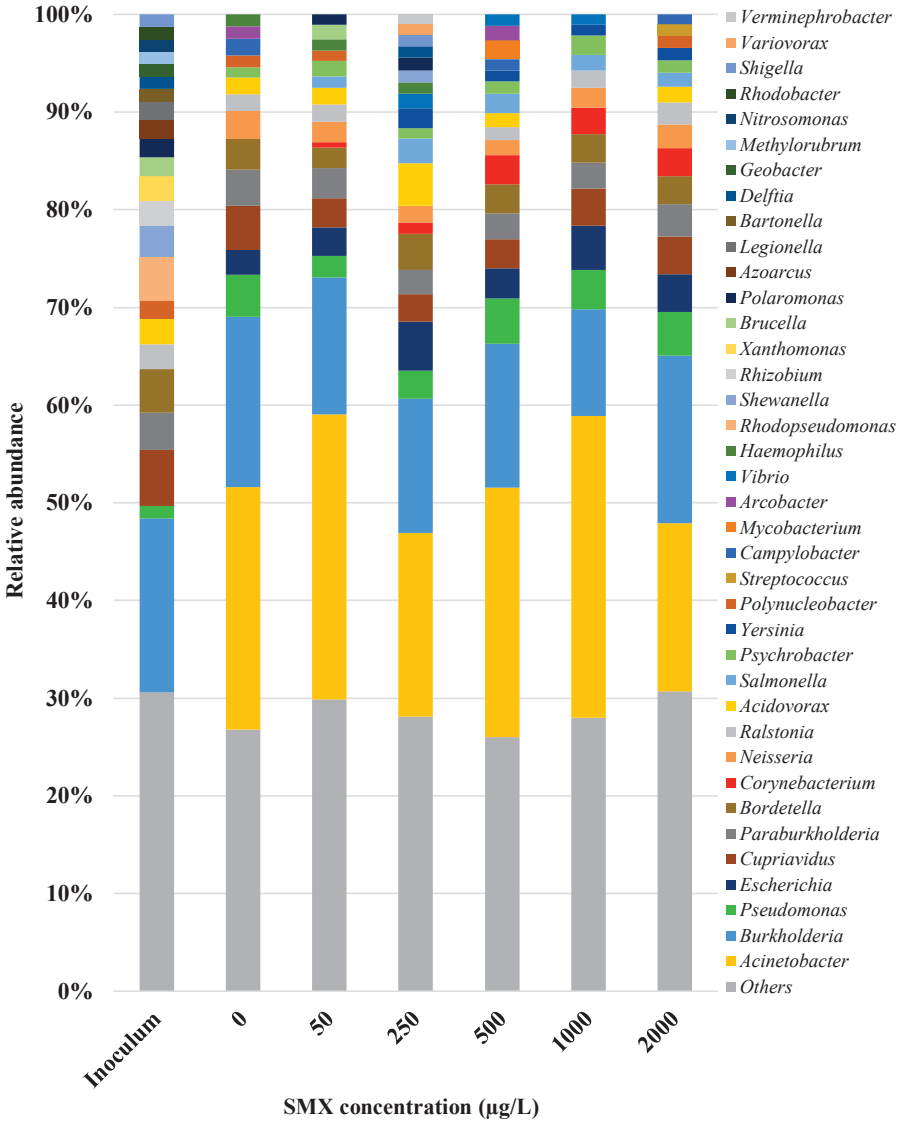


Fig. 5.3. Abundance of the bacterial genera based on the metaproteomic analyses of the inoculum and biomass samples collected on day 25 from the sequential batch reactors fed with different SMX concentrations. The genera that had a contribution ≤1 % are grouped in "Others".

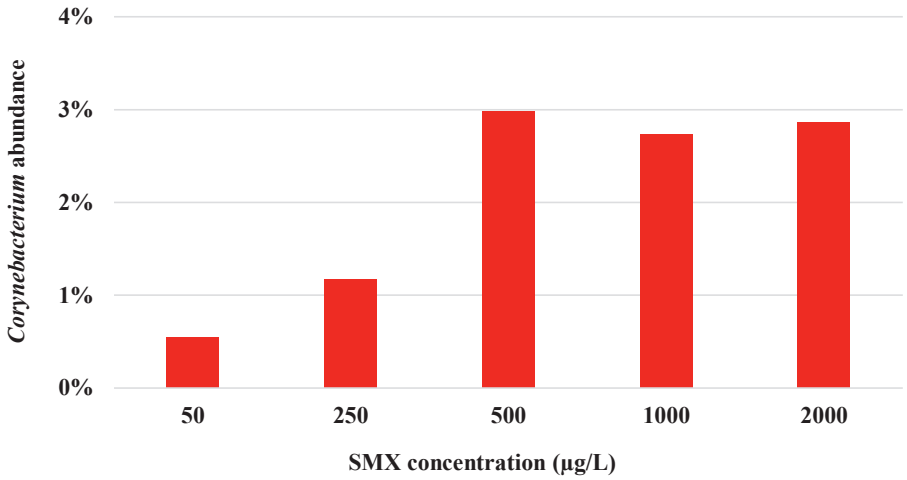


Fig. 5.4. *Corynebacterium* abundance in the microbial community determined through shotgun metaproteomics on day 25 of operation of the sequential batch reactors spiked with different SMX concentrations. In the inoculum and samples from the reactors without SMX, *Corynebacterium* proteins were detected with an abundance below 1 %.

The *Corynebacterium* enzymes isocitrate lyase, aconitate hydratase, malate dehydrogenase, citrate synthase, which are related to the TCA cycle, and enolase, which participates in a step of glycolysis, were differentially expressed in presence of varying SMX concentrations (Table 5.2). These five enzymes are related to central carbon metabolic functions and increased their abundance proportionally to SMX concentrations until 500 µg L^{-1}, decreasing then at 1000 and 2000 µg L^{-1}. The results correlate with the SMX biotransformation yield trend observed in the sequential batch reactors, strengthening the hypothesis that *Corynebacterium* strains might be biotransforming SMX. In fact, *Corynebacterium spp*. have been previously reported as susceptible to SMX, although they had not been linked before to its biotransformation. Yet, such link has been reported for other members of its phylum (*Actinobacteria*) (Reis et al., 2018), such as for the genus *Gordonia*, which belongs to the same order as *Corynebacterium* (*Gordoniaceae*) (Mulla et al., 2018). Alternatively, the increase in expression up to 500 µg L^{-1} could be a response to toxicity events, agreeing with studies reporting that an increase in central metabolic activity can happen under antibiotic stress (Zhang et al., 2021), while the following decrease at the highest concentrations could be attributed to acute SMX or TPs toxicity.

Overall, the abundance increase of *Corynebacterium* DNA and some of its proteins, as well as the overexpression of its central metabolism, suggest that this genus is related to SMX consumption as a source of nutrients (e.g., C or N), energy or as a final electron acceptor. The overexpressed *Corynebacterium* enzymes are related to the TCA cycle and, although their typical substrate-specificity makes unlikely their involvement in the initial biotransformation of SMX, they could have participated in the transformation of smaller metabolites, such as those formed in the last steps of the pterin-conjugation pathway. In fact, some sulfonamide TPs have been reported to be channeled into the TCA cycle (Reis et al., 2020). Differently, the detection of PtO-SMX and N^4-acetyl-SMX in the continuous and sequential batch reactors indicates that a cometabolic SMX biotransformation route was also active on the microbiomes. As described by Nunes et al. (2020), certain microbes can combine multiple SMX resistance mechanisms, such as enzymatic modifications (cometabolism) and energy or nutrient acquisition. Besides, both metabolic and cometabolic processes have been reported to co-occur in the removal of many OMPs (Çeçen et al., 2010; Tran et al., 2013). Therefore, both mechanisms may coexist during SMX biotransformation, possibly due to the action of bacteria from different taxa.

Table 5.2. Differently expressed *Corynebacterium* enzymes detected by shotgun metaproteomics in the sequential batch reactors on day 25. The total and unique number of peptides identified in the study of the proteome samples are listed in the first and second columns. The remaining columns show the Spec values obtained for each protein, which are based on the spectral peptide match counts and allow to compare their relative abundance between samples.

	Total peptides detected	Total unique peptides detected	0 µg/L	50 µg/L	250 µg/L	500 µg/L	1000 µg/L	2000 µg/L
Isocitrate lyase	10	10	0	4	8	13	10	15
Aconitate hydratase	7	6	0	0	1	8	10	2
Malate dehydrogenase	6	6	0	0	2	4	9	5
Enolase	5	5	0	0	2	5	4	1
Citrate synthase	5	4	0	0	2	9	4	3

5.4. CONCLUSION

This chapter highlights the capacity of the heterotrophic sludge to biotransform SMX while still maintaining the high biodegradation levels of the primary substrate. However, under exposure to increasing SMX concentrations, the composition of the heterotrophic microbiota changed. Firstly, *Rhodobacteraceae*, *Comamonadaceae*, *Xanthobacteraceae, Devosiaceae* and *Hyphomicrobiaceae* showed characteristic trends in the continuous bioreactors. Secondly, the abundance of *Corynebacterium* varied in the sequential batch reactors, pointing towards its relevant role to maintain the fitness of the microbial community thanks to the action of some enzymes of its central metabolism. Thus, new insights to understand the SMX biotransformation mechanisms and decipher the key microorganisms and enzymes under aerobic heterotrophic conditions are provided.

Furthermore, the advantages and usefulness of metaproteomics to study OMPs biotransformation mechanisms are proved, providing arguments to the extensive debate raised in the last few years about the benefits and limitations of omic techniques in environmental engineering applications (Achermann et al., 2020; Fenner et al., 2021; David R. Johnson et al., 2015b; Kennes-Veiga et al., 2021a). However, some enzymes that should have been present in the sludge could not be detected, suggesting that further technical developments to lower the identification and quantification thresholds are still required.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

6.1. MAIN OUTCOMES OF THE THESIS

The concern about OMPs emissions into the environment has been increasing in the last decades due to the growing consumption of pharmaceuticals, personal care products, industrial chemicals and many other organic substances. These compounds pose negative effects to both the ecosystem and human health, making it necessary to reduce their concentrations in WWTPs effluents, which are the main source of OMPs discharge to the environment (Margot et al., 2015). Yet, the elimination efficiencies of many OMPs in WWTPs are highly variable or very low. For such reason, research has traditionally focused mostly on finding the most appropriate operational and environmental conditions to improve OMPs removals in WWTPs (Alvarino et al., 2018a). Nonetheless, specific changes in the operational parameters have led to different outcomes in terms of elimination due to their variable effect on the microbial populations and metabolic pathways (Ngo et al., 2020). Furthermore, certain environmental conditions may favor the elimination of some OMPs but, at the same time, hinder that of others (Alvarino et al., 2018b; Harb et al., 2019). Therefore, to establish long-lasting solutions to reduce OMPs concentrations globally in WWTPs effluents, it is necessary to have a deep understanding of the biotransformation mechanisms and to elucidate the bacterial and enzymatic key players.

Most WWTPs consist of an activated sludge system comprising both autotrophic nitrifying and heterotrophic activities (Metcalf & Eddy, 2014). To date, multiple studies are dealing with the role of nitrifiers during OMPs biotransformation, but there is scarce information about the heterotrophic biotransformation mechanisms due to their huge and complex metabolic network (Fischer and Majewsky, 2014). However, previous works have shown their potential to contribute to OMPs removal and reduce the accumulation of TPs formed by nitrifying bacteria (Tran et al., 2013; Wu et al., 2020). Besides, the heterotrophic activity is gaining interest in the development of innovative and more energy-efficient WWTPs (Liu et al., 2020).

Thus, this thesis tries to provide insight and increase the knowledge into the OMPs biotransformation mechanisms in aerobic heterotrophic conditions, aiming to solve the aforementioned questions (**Chapter 1**). In the following sections of this chapter, the main outcomes of this thesis are integrated and jointly discussed.

6.1.1. Are aerobic heterotrophs effective to biotransform OMPs?

The contribution of aerobic heterotrophic microorganisms to the removal of OMPs in activated sludge systems has generally been underestimated. The results shown in **Chapters 3 and 4** prove that heterotrophs can biotransform a range of OMPs with broad physico-chemical properties to extents similar to those reported in the literature for activated sludge and purely nitrifying systems. In fact, for some compounds, such as SMX, DCF and TCS (**Chapter 4**), the heterotrophic biotransformation seems to be better than that of nitrifiers. These results suggest that the A-stages of novel WWTPs, working at high OLRs and short SRTs and operating primarily under heterotrophic conditions (Jimenez et al., 2015), could be able to efficiently and extensively biotransform OMPs before they reach the B-stage, which is proposed to be operated by an anammox consortium (Liu et al., 2020). Additionally, these findings question the need of promoting the activity of autotrophic nitrifiers in WWTPs, which, compared to heterotrophs, are slower growers and possess a less efficient metabolism and a lower diversity of organisms (Holtmann and Sell, 2002; K. Kim et al., 2020). However, the combination of both microbial populations could still be required to reduce the accumulation of some TPs, that may be formed by specific microorganisms but require others for their complete mineralization (Khunjar et al., 2011; Wu et al., 2020).

So far, aerobic heterotrophs are the only microbial population that has proven their capacity to metabolize OMPs (Tran et al., 2013), raising the question about the relevance of metabolism and cometabolism in WWTPs (Alvarino et al., 2018a; Fischer and Majewsky, 2014). However, most previous studies have been performed with pure or enriched cultures and/or using OMPs concentrations considerably higher than those typically reported in WWTPs influents, making it difficult to extrapolate lab results to real-scale plants. In this thesis, seeking to match WWTPs conditions as much as possible, continuous reactors were operated long-term using activated sludge as the inoculum and spiking OMPs in the low μg L^{-1} range. The results proved that cometabolism was the preferential biotransformation mechanism under aerobic heterotrophic conditions and that the fate of OMPs is linked to that of primary substrates (**Chapter 3**). Accordingly, the enzymes with low substrate specificity present in the reactor are responsible for OMPs biotransformation thanks to similarities in the chemical structures of the OMPs and the enzyme's natural substrates.

The biotransformation kinetic constant (k_{biol}) provides information about the biodegradability of OMPs (Han Tran et al., 2017) and, for the same compound, can greatly vary between WWTPs (Helbling et al., 2015; van Bergen et al., 2021). Such variability is generally attributed to changes in operational and environmental conditions that lead to variations in the microbiome and, consequently, in its biotransformation capacity. In **Chapter 3**, it is shown that increases in the intensity of the heterotrophic metabolic activity also lead to changes in k_{biol}, improving OMPs biotransformation. Thus, k_{biol} is not only dependent on the physico-chemical properties of the OMPs and the conditions applied to the WWTPs, but also on the metabolic activity of the biomass. This finding implies that the management of the organic substrates and the OLR can be efficient tools to control the microbial activity and, subsequently, OMPs biotransformation. Nonetheless, the changes observed in k_{biol} proved to be compound-specific, suggesting that variations in the metabolic activity carry over unevenly across the biotransformation rates of the OMPs. Besides, if the HRT applied is high enough, improved biotransformation rates may not translate into better biotransformation extents.

Overall, aerobic heterotrophic microorganisms can extensively biotransform OMPs through cometabolism and influence the biotransformation rate through changes in k_{biol} caused by variations in the metabolic activity.

6.1.2. Transformation products as a tool to identify key enzymatic activities

Biotransformation reactions occur thanks to the versatile catalytic activity of the enzymes involved in the biodegradation of primary substrates. Identifying such key enzymes is a complex task that is still in its early stages (Achermann et al., 2020; Krah et al., 2016; Wicker et al., 2016; Zhou et al., 2015) and, in aerobic heterotrophic conditions, there are no conclusive studies about the enzymatic activities involved in OMPs biotransformation. Aiming to fill in that gap, several experiments with heterotrophic sludge were carried out in this thesis through two different methodologies : *(i)* TPs and *(ii)* -omic analyses.

TPs analysis has been traditionally challenging due to the low OMPs concentrations found in environmental samples and the complex matrices where they are present (L. Zhang et al., 2013). However, TPs studies aiming at understanding their ecotoxicity and risk to the environment and the biotransformation limitations of OMPs have become more frequent in recent times thanks to major advances in analytical techniques (Fenner et al.,

2021; Gulde et al., 2016). Additionally, TPs structure analysis can be used as a prediction tool to determine the main enzymatic activities involved in OMPs biotransformation (**Chapter 1**). If primary substrates and TPs are known, it is possible to perform educated guesses about the enzymes carrying out the process.

Applying this methodology, in this thesis (**Chapter 4**) it was possible to identify mono- and dioxygenases, dehydrogenases, hydrolases and transferases as some of the main enzymatic activities likely responsible for OMPs biotransformation under heterotrophic conditions. The reactions catalyzed by these enzymes involve oxidation, hydrolysis and conjugation steps. Oxidation, which is frequently an initial biotransformation step for many OMPs in activated sludge systems, was the main biotransformation reaction observed. Among oxidative steps, hydroxylation was the most predominant, being detected for multiple compounds, although oxidation through deamination, demethylation and dehydrogenation reactions was also determined. Interestingly, for some OMPs, different TPs were identified, suggesting that sequences of transformation reactions may take place at different functional groups. Besides, some of the TPs detected have been described as active metabolites with long-half lives, highlighting the need to elucidate their fate in WWTPs and perform ecotoxicity analyses.

Identifying TPs can be a challenging task because OMPs can undergo a huge array of biotransformation reactions, leading to a tremendously large number of possible TPs. Therefore, the application of modeling tools, such as the EAWAG-PPS (EAWAG-BBD/PPS) (**Chapter 4**), which are based on biotransformation rules supported by multiple literature studies, have proven to be of great aid to create lists of suspect TPs. Their use, along with lab experiments that serve as a confirmation method, is recommended to build pathway knowledge and predict the formation and toxicity of TPs.

Overall, this thesis proved that TPs analysis is a useful tool to unravel relevant enzymes taking part in biotransformation reactions. However, this methodology alone does not allow to obtain direct and complete confirmation of the responsible enzymatic activities. Thus, it is advisable to combine the TPs results with those from other analytical methods, such as *in vitro* assays, or omic techniques, as discussed in the following section.

6.1.3. Omic techniques as a tool to identify key microbial and enzymatic players

Metagenomic, metatranscriptomic and metaproteomic techniques are evolving rapidly and meta-omics association studies have started to be used to identify key

microbial and enzymatic players involved in OMPs biotransformation pathways (Achermann et al., 2020; Du et al., 2017; Fenner et al., 2021). Their potential is based on the fact that introducing OMPs in a specific environment affects the structure of the microbiome, leading to variations in the expression of genes and gene products and allowing to find biomarkers that help understand and predict the fate of OMPs in WWTPs. However, the application of this approach still raises some doubts; mainly because *(i)* many false positives leading to non-causal correlations can be easily generated and *(ii)* it is still necessary to confirm that the overexpression of gene products happens even when OMPs are added at trace level concentrations (Fenner et al., 2021).

In **Chapter 5**, the characteristic trends followed by five bacterial families belonging to the phylum *Proteobacteria* and the key role of *Corynebacterium* during the heterotrophic biotransformation of SMX could be determined thanks to variations in their abundance measured through the application of DNA metabarcoding and metaproteomic techniques. Besides, a differential expression of five enzymes involved in central metabolic functions of *Corynebacterium* was also observed, suggesting its relevance during SMX biotransformation. Interestingly, these findings point towards the importance of the TCA cycle enzymes during OMPs biotransformation, which has not been reported before. It is unlikely that these enzymes participate in the initial biotransformation steps given the usual size and structure of OMPs, but they could be involved in the conversion of smaller TPs formed after several biotransformation steps, such as those belonging to the SMX-pterin conjugation pathway.

Therefore, results highlight that omic techniques are useful to unravel biotransformation pathways and identify relevant microorganisms and enzymatic activities. Nonetheless, some enzymes that should have been present in the sludge could not be detected through metaproteomics (**Chapter 5**), possibly due to their low abundance compared to that of housekeeping or central metabolism proteins, which shows that technical developments to improve enzyme identification and quantification are still required. Additionally, in **Chapter 5**, *Corynebacterium* abundances differed between the results from DNA metabarcoding and metaproteomic analyses. Thus, omic techniques representing expressed gene products at a given time, such as metatranscriptomics or metaproteomics, may be more suitable to find causal links between OMPs biotransformation and microbiological data.

6.2. MAIN GAPS AND FUTURE PERSPECTIVES

There are several research areas and knowledge gaps that need to be addressed to better understand biological processes in WWTPs and improve OMPs removal. Cometabolism has been identified as the main mechanism driving OMPs biotransformation; however, further research is required to determine where the boundary between metabolism and cometabolism lies (Nsenga Kumwimba and Meng, 2019; Tran et al., 2013). To this end, experiments with increasing OMPs doses, along with -omics and TPs analyses, could be carried out. These experiments would also allow determining the threshold where inhibitory events appear and provide information about changes in biotransformation routes at higher OMPs concentrations (Jia et al., 2020; Wegner et al., 2015), which could be particularly useful for highly concentrated streams, such as hospital or industrial wastewaters. Moreover, the contribution of secondary biological activities of the sludge during the cometabolic biotransformation of OMPs remains unclear (Gonzalez-Gil et al., 2021). The more recent use of radiolabeling-based approaches, which consist of the addition of a labeled substrate to the experimental environment along with an analysis of label incorporation into TPs, biomarkers, enzymes or cells, has been pointed out as a promising technique to solve these questions. Yet, its application with OMPs present at low levels remains challenging (Falås et al., 2018; Fenner et al., 2021).

Insight on the role and origin of the enzymes responsible for OMPs biotransformation, as well as on the conditions that increase their expression, is necessary to implement operational strategies in WWTPs that enhance their abundances and activities (Fischer and Majewsky, 2014; Krah et al., 2016). Such knowledge could eventually allow to select enzymes as a bioremediation technique to target specific compounds, particularly for the treatment of well-defined waste streams. It would offer a more direct, controlled and defined alternative than conventional WWTPs, which comprise a huge consortium of microorganisms and enzymes and require the control of cell growth, substrate transport to the cell and sorption, among others (Stadlmair et al., 2018). Nonetheless, the application of this technique in real systems is still a long way off for several reasons: *(i)* the isolation of target enzymes from WWTPs has not been achieved yet, *(ii)* the purchase of enzymes is too costly and presents limitations of large-scale production and *(iii)* setting up cooperation between multiple enzymes in WWTPs

and dealing with enzymatic inactivation, stability and specificity is still a complex task (Feng et al., 2021; Langbehn et al., 2021; Stadlmair et al., 2018).

To date, although some approaches have been developed and applied to identify the enzymes involved in OMPs biotransformation, they still present multiple limitations. For instance, enzymatic *in vitro* assays with cell-free lysates require further research on the extraction procedure to preserve the indigenous enzymatic activities present in the sludge, including the application of pretreatments and buffer additives (Krah et al., 2016). Moreover, it is necessary to improve the understanding of the circumstances in which *in vitro* assays are fully comparable to real biological processes and to evaluate the adequacy of applying cofactors or enzymatic inhibitors, that may boost or hinder specific enzymes and help to confirm their activity towards OMPs (Gonzalez-Gil et al., 2019b). To facilitate the link between enzymes and biotransformation pathways, the detection, quantification and assessment of TPs require the continuous improvement of modeling tools and analytical techniques that allow sensitive and untargeted TPs characterization in short time frames even when reference standards are not available (Nguyen et al., 2021). In the past decades, high-resolution mass spectrometry has resulted in notorious advances to determine low OMPs and TPs concentrations even in complex mixtures. More recently, compound-specific isotope analysis is being developed, although its application to environmental OMPs concentrations remains a challenge. This technique can potentially help to determine the biotransformation extent and reaction mechanisms of OMPs and elucidate different origins of the same TPs thanks to the usual enzymatic preference for molecules with light isotopes, which leads to changes in the isotopic ratios of the biotransformed compounds (Fenner et al., 2021). Finally, omics techniques need to reduce their costs to broaden their accessibility and their results must be supported with powerful statistical tools to facilitate the search of causal relationships between enzymes and OMPs biotransformation (Achermann et al., 2020; David R. Johnson et al., 2015b).

As a result of the abovementioned limitations, understanding the influence of environmental and process conditions and confidently obtaining mechanistic insights about the biotransformation of OMPs in WWTPs by directly and exclusively applying any of the well-established approaches is still not possible. However, a combination of all methodological approaches is the best way to overcome some of the individual drawbacks and formulate sound hypotheses about the microorganisms and enzymes participating in OMPs biotransformation, in line with previous studies (Achermann et al.,

2018b, 2020; Zhao et al., 2020) and the results of this thesis (**Chapter 5**). Thus, to maximize OMPs removal is essential an interdisciplinary contribution from the scientific community, covering the fields of environmental engineering, analytical chemistry, molecular biology and data sciences, as well as validating laboratory findings in full-scale mixed cultures with a wide range of OMPs present at environmentally relevant concentrations.

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LIST OF PUBLICATIONS

PEER REVIEWED JOURNALS

Kennes-Veiga, D. M.[a], Gonzalez-Gil, L.[b], Carballa, M. [a], Lema, J. M. [a] (2021) Enzymatic cometabolic biotransformation of organic micropollutants in wastewater treatment plants: A review. Bioresource Technology (Elsevier). 344B, 126291. ISSN: 0960-8524, https://doi.org/10.1016/j.biortech.2021.126291. Impact factor (2020): 9.642, Q1.

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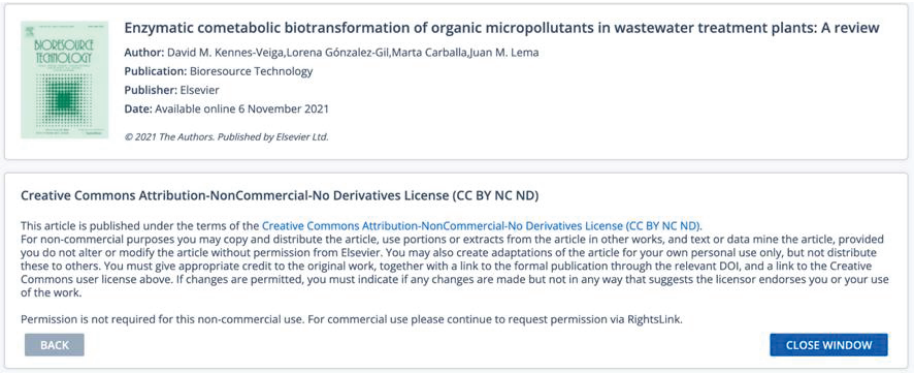
Chapter 1 is based on this publication. The author of this thesis contributed to the conceptualization, investigation, formal analysis and writing process of this article.



Enzymatic cometabolic biotransformation of organic micropollutants in wastewater treatment plants: A review

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Kennes-Veiga, D. M.[a], Gonzalez-Gil, L.[b], Carballa, M. [a], Lema, J. M. [a] (2020) The organic loading rate affects organic micropollutants' cometabolic biotransformation kinetics under heterotrophic conditions in activated sludge. Water Research (Elsevier), 189, 116587. ISSN: 0043-1354, https://doi.org/10.1016/j.watres.2020.116587. Impact factor (2020): 11.236, Q1.

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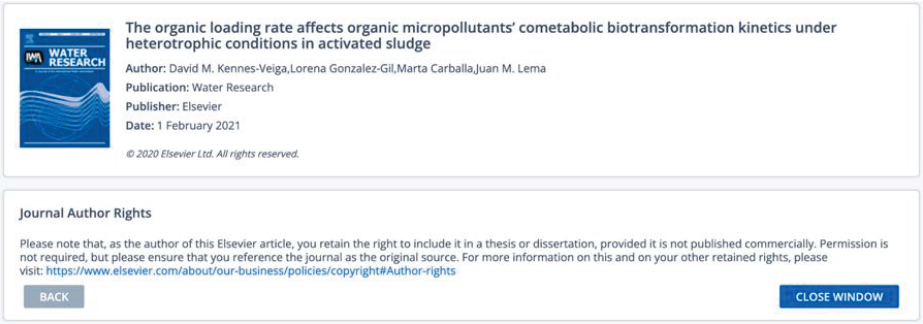
Chapter 3 is based on this publication. The author of this thesis contributed to the conceptualization, investigation, formal analysis and writing process of this article.



Kennes-Veiga, D. M.[a], Vogler, B.[b], Fenner, K.[b,c,d], Carballa, M.[a], Lema, J. M.[a] (2021) Heterotrophic enzymatic biotransformations of organic micropollutants in activated sludge. Science of the Total Environment (Elsevier), 780, 146564. ISSN: 0048-9697, https://doi.org/10.1016/j.scitotenv.2021.146564. Impact factor (2020): 7.963, Q1.

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Chapter 4 is based on this publication. The author of this thesis contributed to the conceptualization, investigation, formal analysis and writing process of this article.

Kennes-Veiga, D. M.[a], Trueba-Santiso, A.[a], Gallardo-Garay, V. [a], Sabela, S.[b], Carballa, M. [a], Lema, J. M. [a] (2022) Sulfamethoxazole triggers specific enzymatic activities under aerobic heterotrophic conditions: A metaproteomic approach. Environmental Science and Technology (American Chemical Society). ISSN: 1520-5851. *Under review*. Impact factor (2020): 9.028, Q1.

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Chapter 5 is based on this publication. The author of this thesis contributed to the conceptualization, investigation, formal analysis and writing process of this article.

Kennes-Veiga, D. M., Balboa, S., Lema, J. M., Carballa, M. (2022) Identifying key players involved in sulfamethoxazole and ibuprofen biotransformation under aerobic heterotrophic conditions. *In preparation*.

CONFERENCE PROCEEDINGS

Oral contributions

Kennes-Veiga, D. M., Gónzalez-Gil, L., Carballa, M., Lema, J. M. Cometabolic biotransformation of organic micropollutants under aerobic heterotrophic conditions. 11th Micropol & Ecohazard Conference 2019. Seoul, South Korea, October 2019.

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Kennes-Veiga, D. M., Gónzalez-Gil, L., Carballa, M., Lema, J. M. Cometabolic microbial activity affects organic micropollutants biotransformation kinetics. 5th IWA Specialized International Conference "Ecotechnologies for Wastewater treatment (EcoSTP)". Milan, Italy, June 2021.

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Trueba-Santiso, A., **Kennes-Veiga, D. M.**, Gallardo-Garay, V., Quitón-Tapia, S., Lema, J. M. Metaproteomics and its potential for the study of biological processes in microbial mixed communities. V Simposio Investigación en Tecnologías Ambientales, CRETUS. Santiago de Compostela, Spain. September, 2021.

Poster contributions

Kennes-Veiga, D. M., Carballa, M., Lema, J.M. Biotransformación enzimática cometabólica de microcontaminantes orgánicos en condiciones aerobias heterótrofas. II Simposium Investigación en Tecnologías Ambientales, CRETUS. Santiago de Compostela, Spain. May, 2018.

Kennes-Veiga, D. M., Gónzalez-Gil, L., Carballa, M., Lema, J. M. Estudio de la biotransformación cometabólica de microcontaminantes en condiciones aerobias heterótrofas. Simposio Novedar "Presencia y eliminación de microcontaminantes en agua". Santiago de Compostela, Spain, June 2019.

The high consumption of organic micropollutants (OMPs) in modern societies is an issue of increasing concern due to the risks they pose to the aquatic environment. Therefore, it is essential to understand and identify the mechanisms, biotransformation pathways and key microorganisms and enzymatic activities involved in their removal processes in wastewater treatment plants (WWTPs). In this thesis, the cometabolic biotransformation of OMPs under aerobic heterotrophic conditions is assessed, seeking to expand the current knowledge and facilitate the design and optimization of WWTPs to reduce their concentrations.