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ESTUDIO TÉCNICO Y BIOLÓGICO DE UN SISTEMA MBBR CON DIGESTIÓN BIFÁSICA EN LÍNEA DE FANGOS PARA LA ELIMINACIÓN DE CONTAMINANTES EMERGENTES Y CONTROL DE NUTRIENTES EN AGUAS RESIDUALES URBANAS

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TECHNICAL AND BIOLOGICAL STUDY OF A MBBR SYSTEM WITH TWO-STAGE ANAEROBIC DIGESTION IN THE SLUDGE LINE FOR THE ELIMINATION OF EMERGING POLLUTANTS AND NUTRIENT CONTROL IN URBAN WASTEWATER

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ABREVIATURAS/ABBREVIATIONS

A^2O	anaerobic/anoxic/aerobic aneróbico/anóxico/óxico
AcD	acidogenic digester digestor acidogénico
AcS	acidogenic sludge fango acidogénico
AD	anaerobic digestion digestión anaeróbica
AIAP(s)	anti-inflammatory and/or analgesic pharmaceutical(s) fármaco(s) analgésico(s) y/o antiinflamatorio(s)
ALK	alkalinity alcalinidad
AOA	ammonia-oxidizing archaea arquea oxidadora de amonio
AOB	ammonia-oxidizing bacteri bacteria oxidadora de amonio
BD	biofilm density
BFSS	biofilm fixed suspended solids biomasa adherida
BFVSS	biofilm fixed volatile suspended solids biomasa adherida volátil
BNR	biological nutrient removal eliminación de nutrientes biológica
BOD_5	biological oxygen demand demanda biológica de oxígeno
BPR	biogas production rate ratio volumétrico de producción de biogás
CAS	conventional active sludge fangos activos convencionales
COD	chemical oxygen demand demanda química de oxígeno
DDD	defined daily dose dosis definida por día

DHD	DDD unit per 1000 inhabitant per day Dosis definida por día por cada 1000 habitantes
DM	dry matter
DMSO	Dimethyl sulfoxide
DNA ADN	desoxyribonucleic acid ácido desoxirribonucleico
DO	dissolved oxygen oxígeno disuelto
D_{ow}	octanol-water distribution coefficient, pH-dependent coeficiente de distribución octanol-agua dependiente del pH
EDAR	estación de depuradoras de aguas residuales
EQS	Environmental Quality Standards
EQSD	Environmental Quality Standards Directive
ERA	environmental risk assessment
F/M ratio	food/microorganism ratio carga másica
GWD	Ground Water Directive
HRT TRH	hydraulic retention time tiempo de retención hidráulico
HTS	high-throughput sequencing
IFAS	integrated fixed film activated sludge
K_{biol}	biodegradable rate loss constant coeficiente de biodegradabilidad
K_d	sorption potential coefficient coeficiente de sorción experimental
K_{ow}	octanol-water partition coefficient coeficiente de partición octanol-agua
MAD	mesophilic anaerobic digestion digestión anaeróbica mesofílica
MBBR	moving bed biofilm reactor biorreactor de lecho móvil

MBMBR	moving-bed membrane bioreactors biorreactor de membrana con lecho móvil
MBR(s)	membrane bioreactor(s) biorreactor de membrana(s)
MD	methanogenic digester digestor metanogénico
MDS	non-metric multidimensional scaling escala multidimensional no métrica
ML	daily measured mass load carga másica diaria
ML-F/M	mixed liquor food/microorganism ratio carga másica del licor mezcla
MLR	mixed-liquor recycle recirculación interna del licor mezcla
ML-SRT	mixed liquor sludge retention time tiempo de retención celular del licor mezcla
MLSS	mixed liquor suspended solid sólidos suspensos del licor mezcla
MLVSS	mixed liquor volatile suspended solids sólidos suspensos volátiles del licor mezclan
NLR	nitrogen loading rate carga másica de notrógeno total
NRR	total N removal rate tasa de eliminación de nitrógeno total
OLR	organic loading rate carga másica de materia orgánica
ORR	organic removal rate tasa de eliminación de materia orgánica
OT	operational temperature temperature de operación
OTU(s)	Operational taxonomic unit(s) Unidad(es) taxonómica(s) operacional(es)
p.i.e.	per inhabitant equivalent habitantes equivalentes

PAO(s)	polyphosphate accumulating organism(s) microorganismos acumuladores de fósforo
PCR	polymerase chain reaction reacción en cadena de la polimerasa
PEC	predicted environmental concentration concentración medioambiental predicha
PhAC(s)	pharmaceutically active compound(s) compuestos farmacéuticamente activos
PNEC	predicted no-effect concentration concentración predicha que no causa efecto tóxico
PLR	phosphorous loading rate carga másica de fósforo
PRR	total P removal rate tasa de eliminación de fósforo
PS	primary sludge fango primario
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative Real-Time Polymerase Chain Reaction Reacción en cadena de la polimerasa cuantitativa en tiempo real
RAS	returned activated sludge recirculación de fangos secundarios
RE(s)	removal efficiency(ies) tasa de eliminación
RQ	risk quotient
rRNA ARNr	Ribosomal RNA ARN ribosómico
RSD	relative standard deviation desviación estandar relativa
SCADA	supervisory control and data acquisition
SMP	specific methane production produccion de metano específica

SRT	solid retention time tiempo de retención de sólidos o edad del fango
SVI	sludge volumetric index índice volumétrico de fangos
TAD	termophilic anaerobic digestion digestión anaeróbica termofílica
TGGE	temperature Gradient Gel Electrophoresis Electroforesis en gel con gradiente de temperatura
ThS	thickened sludge fango espesado
TN	total nitrogen nitrogeno total
TP	total phosphorus fósforo total
TS	total solid sólidos totals
TSS	total suspended solid sólidos totals suspendidos
TV	total volatile sólidos volátiles totals
VFA(s)	volatile free acid(s) ácido(s) graso(s) volátil(es)
VSR	volatile solid removal eliminación de sólidos volátiles
WAS	waste activated sludge fangos secundarios en exceso
WFD	Water Framework Directive
WWTP(s)	wastewater treatment plant(s)

RESUMEN/ABSTRACT

Resumen

Durante las últimas décadas, la producción de aguas residuales ricas en nutrientes con una amplia gama de contaminantes emergentes como son productos de cuidado personal, fragancias sintéticas y especialmente los compuestos farmacéuticamente activos (PhACs) ha aumentado continuamente en todo el mundo debido al crecimiento exponencial de las poblaciones urbanas, al consumo doméstico y al crecimiento de la producción industrial. Hoy en día, las plantas de tratamiento de aguas residuales urbanas (EDARs) convencionales se enfrentan a numerosos problemas a la hora de cumplir los estándares más estrictos en términos de vertido de nutrientes y eliminación de contaminantes emergentes. En este sentido, el aumento de la eutrofización de las aguas superficiales receptoras, así como la presencia continua de contaminantes orgánicos (p.ej., PhACs) en las aguas residuales tratadas y fangos digeridos, son temas de creciente preocupación en todo el mundo. Además, varios estudios han demostrado que los PhACs pueden acumularse en el suelo, las aguas subterráneas y las porciones comestibles de las plantas regadas con aguas residuales tratadas, lo que representa para el ser humano una importante exposición a los PhACs con posibles implicaciones para la salud. Para dar solución a estos problemas, en la presente Tesis Doctoral se ha estudiado la presencia, el destino y la eficiencia de eliminación (RE) de 27 PhACs en una planta de tratamiento de aguas residuales a escala piloto operada con procesos convencionales y avanzados en diferentes fases experimentales. Esta investigación se planteó desde un punto de vista multidisciplinar, en busca de vínculos entre la eficacia de la remoción de los PhACs, el rendimiento de sistemas de tratamiento, los parámetros de operación, y la estructura de las comunidades microbianas en la biomasa, tanto en la línea de agua como en la línea de fangos de la planta piloto.

La planta piloto utilizada es una copia a escala (1:25.000) de la EDAR Murcia Este (Murcia, España) que trata hasta 6 m^3d^{-1} de las aguas residuales procedentes de esta depuradora. La investigación se dividió en tres períodos experimentales (Fase I, II y III) en la línea de aguas. En las Fases I y II se operó durante 208 días con un sistema convencional de eliminación biológica de nutrientes en configuración A^2O (anaeróbico/anóxico/aeróbico), y con diferentes parámetros operacionales entre fases; mientras que en la Fase III, el biorreactor A^2O se transformó en un biorreactor híbrido de lecho móvil (sistema IFAS “Integrated Fixed-Film Activated Sludge”) rellenando el 50% de la cámara aeróbica con el soporte plástico AnoxKaldnes K5. En la línea de fangos, el fango primario y secundario producido por la línea de aguas fue espesado y tratado por

dos digestores en serie usando el sistema no convencional de digestión anaeróbica bifásica (MAD). La digestión bifásica fue operada durante dos fases experimentales (Fase I y II) con una duración de 104 días cada una, utilizando dos conjuntos diferentes de tiempos de retención hidráulico (TRH) para los digestores acidogénicos (AcD) y metanogénicos (MD) (Fase I, 2 y 12 días; y Fase II, 5 y 24 días, en AcD y MD, respectivamente). Se realizaron análisis qPCR y de secuenciación masiva (Illumina MiSeq) para cuantificar e investigar la estructura, diversidad y dinámica de poblaciones de las comunidades de bacterias, arqueas y hongos en el fango activado del biorreactor A^2O y el fango digerido del AcD y MD. Se utilizaron análisis multivariantes y coeficientes de correlación de Spearman en busca de vínculos significativos entre las REs de los PhACs seleccionados, las abundancias de los grupos microbianos en el biorreactor y los digestores, y los cambios de las variables ambientales/operativas de los biorreactores A^2O, A^2O-IFAS y los digestores de la AcD y MD.

En la línea de agua, los resultados mostraron que el sistema A^2O mejoró las REs de materia orgánica, nutrientes y 5 PhACs cuando se operó con mayor concentración de biomasa (MLSS) y menor F/M ratio, en la Fase II; en consecuencia, se encontraron correlaciones positivas entre las REs de estos PhACs y varias variables operativas/ambientales (MLSS, F/M ratio, concentración del influente $N\text{-}NH_4^+$ y tasa de eliminación de nitrógeno total). Sin embargo, cuando el biorreactor operó con el sistema A^2O-IFAS en la Fase III, las REs de materia orgánica, nutrientes y de 8 y 5 PhACs mejoraron significativamente en comparación con el sistema convencional A^2O (Fase I y II, respectivamente). Además, estas mejoras de rendimiento del sistema A^2O-IFAS se produjeron con un menor coste operativo. En la línea de fangos, se encontraron los rangos de TRHs óptimos para la digestión bifásica en función de la producción de ácidos grasos volátiles (VFA) y metano en ambos digestores; este hecho fue corroborado por las fuertes interrelaciones encontradas entre las variables operacionales y de rendimientos, y la abundancia relativa de grupos de bacterias y arqueas. Finalmente, la extensión del TRH de 12 días (Fase I) a 24 días (Fase II) en la DM se vinculó significativamente con una mejor eliminación de 7 PhACs.

Los gráficos de escalado multidimensional no métrico (MDS) y el análisis de biota-ambiente (BIO-ENV) revelaron fuertes correlaciones entre la estructura de las comunidades de bacterias y arqueas del biorreactor A^2O de la línea de aguas, y los digestores AcD y MD de la línea fangos, con los cambios de las variables operacionales/ambientales de cada biorreactor entre las fases experimentales, que

posteriormente se correlacionaron con diferencias en las tasas de eliminación de nutrientes en la línea de aguas y tasas de producción de biogás en la línea de fangos. El análisis BIO-ENV y MDS también estableció sólidas interconexiones entre los REs de 11 PhACs con los cambios de las abundancias relativas de diferentes familias de bacterias y géneros de arqueas en la línea de aguas. Del mismo modo, se detectaron correlaciones sólidas entre los REs de 6 PhACs y las abundancias relativas de varios grupos de bacterias y arqueas en los AcD y MD de la línea de fangos. Estas interrelaciones señalaron su potencial relación con los procesos de biodegradación/biotransformación de estos compuestos xenobióticos en los sistemas de tratamiento de fangos y aguas residuales urbanas.

Abstract

During the last decades, the production of nutrient-rich wastewaters with a wide range of emerging contaminants such as personal care products, ultraviolet filters, synthetic fragrances and especially the active pharmaceutically compounds (PhACs) has been continuously increasing worldwide due to the exponential growth of urban populations, household consumption and industrial production. Nowadays, conventional wastewater treatment plants (WWTPs) have demonstrated many shortcomings to face more stringent discharge standards in terms of nutrient release and emerging contaminants removal. In this sense, the continues presence of different trace organic contaminants such as PhACs in treated urban wastewaters and treated sewage sludge is an issue of growing concern worldwide. Moreover, several studies have shown that PhACs can be accumulated into the soil, groundwaters and edible portions of plants irrigated with treated wastewater, representing an important exposure of pharmaceutical to humans, with potential health implications. To overcome these problems, the present PhD Thesis has studied the occurrence, fate and removal efficiency (RE) of 27 PhACs in a pilot-scale wastewater treatment plant operated with conventional and advanced processes in different experimental phases. The investigation was developed under a multidisciplinary approach, in search of interlinkages among the removal efficiencies of PhACs, the performance of the treatment system, the changes of operational parameters, and the shifts of the microbial communities' structure in the sludge, in both the water line and sludge line of the pilot-scale plant.

The pilot-scale plant is a scale-copy (1:25,000) of WWTP Murcia Este (Murcia, Spain) and treats up to 6 m^3d^{-1} urban wastewater from the WWTP Murcia Este. The investigation was divided into three experimental phases (Phase I, II and III) in the water line. Phases I and II operated 104 days each with a conventional biological nutrient removal system (anaerobic/anoxic/aerobic, A^2O), and with different operational parameters between phases; while in Phase III, the A^2O bioreactor was transformed into a moving bed hybrid bioreactor ("integrated fixed-film activated sludge" IFAS system) filling the aerobic chamber 50% with the carrier AnoxKaldnes K5. In the sludge line, the primary and secondary sludge produced by the water line of the A^2O system was thickened and treated by two digesters in series using the no conventional two-stage mesophilic digestion system (MAD). The two-stage MAD system was long-term operated during the first two experimental phases (Phase I and II), using two different

sets of hydraulic retention times (HRTs) for the acidogenic (AcD) and methanogenic (MD) digesters (phase I, 2 and 12 days; and phase II, 5 and 24 days, in AcD and MD, respectively). Quantitative polymerase chain reaction (qPCR) and Illumina MiSeq sequencing analysis were used to quantify and to investigate the structure, diversity and population dynamics of bacteria, archaea and fungi communities in the activated sludge of the bioreactor and digested sludge of the AcD and MD. Multivariate analyses and Spearman correlation coefficients were used in search of significant links among the REs of the selected PhACs, the abundances of the microbial groups in the bioreactor and digesters, and the changes of environmental/operating variables in the A^2O, A^2O-IFAS bioreactors and two-stage anaerobic digesters.

In the water line, the results showed that the A^2O system improved the RE of organic matter, nutrients and 5 PhACs when the biomass concentration (MLSS) was improved and the F/M ratio was reduced in Phase II; accordingly, positive correlations were found among the REs of these PhACs and several operational/environmental variables (MLSS, F/M ratio, $N\text{-}NH_4^+$ influent concentration and total nitrogen removal rate). However, when the pilot-scale bioreactor operated with A^2O-IFAS system in the experimental Phase III, the REs of organic matter, nutrients and especially 8 and 5 PhACs was significantly improved compared to the A^2O system (Phase I and II, respectively). Besides, these performance improvements of the A^2O-IFAS system occurred with a lower operational cost. In the sludge line, the optimum HRT ranges for an efficient two-stage MAD was found, based in the production of volatile free acid (VFA) and methane in both digesters, confirmed by the strong interlinkages found among operational/performance variables and the relative abundances of bacterial and archaeal groups. Finally, the extension of the HRT from 12 (Phase I) to 24 days (Phase II) in the MD was significantly linked with an improved removal of 7 PhACs.

Non-metric multidimensional scaling (MDS) plots and Biota-environment (BIO-ENV) analysis revealed strong correlations between the bacteria and archaea communities' structure in the A^2O bioreactors in the water line, and the AcD and MD in the sludge line with the shifts of the operational/environmental variables of each bioreactor among the experimental phases, which were subsequently correlated with differences in the efficiency of nutrient removal in the water line and methane production in the sludge line. The BIO-ENV and MDS analysis also established robust interlinkages between the REs of 11 PhACs with the shifts of the relative abundances of different families of *Bacteria* and genera of *Archaea* in the water line, while robust correlations

were also detected among the REs of 6 PhACs and the relative abundances of several bacterial and archaeal groups in the AcD and MD of the sludge line. These interlinkages pointed out their potential involvement in the biodegradation/biotransformation of these xenobiotics' compounds in the wastewater and sludge treatment processes.

I. GENERAL INTRODUCTION

1. Pharmaceutically active compounds as emerging contaminants

Due to the higher sensitivity of analytical methods and continuous improvement in the instrumentation, pharmaceutically active compounds (PhACs) have been detected in many environmental matrixes and compartments such as surface and groundwater, soils, biota and edible part of agricultural plants (aus der Beek et al., 2016; Christou et al., 2017; de Santiago-Martín et al., 2020; Gogoi et al., 2018; L. Ma et al., 2018; Petrie et al., 2015; Wilkinson et al., 2017; Wu et al., 2015). In this sense, there is growing concern worldwide about the potential adverse effects that the presence of PhACs have on the environment and human health, a fact reflected by the exponential increase in the number of scientific publications on this topic in the last two decades (aus der Beek et al., 2016; COM(2019) 128, 2019; Daughton, 2016).

After ingestion, the PhACs undergo metabolic processes in the organism where a significant fraction is excreted as active substances in the urine and faeces (generally between 30-90% of orally administered drugs) into raw sewage and wastewater treatment systems (Al Aukidy et al., 2012; Jjemba, 2006; Lienert et al., 2007; Verlicchi et al., 2014). The municipal wastewater treatment plant (WWTP) effluents (water and sewage sludge) are discharged into different water bodies or agricultural soils due to the reuse of reclaimed water for irrigation or the application of sewage sludge as amendment organic biosolids. Therefore, body excretion and WWTP effluents are recognized as the main sources of PhACs in the environment (Gogoi et al., 2018; Jelić et al., 2012; Krzeminski et al., 2019; Verlicchi et al., 2012; Verlicchi and Zambello, 2015).

The PhACs can persist in the environment or otherwise bioaccumulate in plants and wildlife due to the constant consumption and continuous release. Many studies have shown significant negative effects in the metabolic, immune and reproductive system in fish residing in treated wastewater effluent-impacted sites (Dzieweczynski et al., 2016; Galus et al., 2013; Lacaze et al., 2019; McCallum et al., 2019, 2017; Mehdi et al., 2018), while a few authors did not find short-term biological effects in zebrafish (Angeles et al., 2020) or *Poecilia reticulata* (guppy) (Zaibel et al., 2019). The veterinary use of the anti-inflammatory drug diclofenac in cattle, produced a serious damage in at least three endemic Gyps vulture species in South Asia, besides similar effects occurred in other scavenging birds outside of Asia (He et al., 2017). Schwaiger et al. (2004) demonstrated that prolonged exposure in environmentally concentration of diclofenac leads to deterioration of the general health conditions of rainbow trout fish.

Furthermore several studies have shown that drugs can be accumulated into edible portions of plants irrigated with treated wastewater and/or cultivated in soils amended with sewage biosolids, these represent an important exposure of pharmaceuticals to humans, with potential health implications (Ben Mordechay et al., 2018; Christou et al., 2017; Paltiel et al., 2016; Wu et al., 2015). On the other hand, Prosser and Sibley (2015) estimated the human health risks of individual PhAC in edible plant tissue and concluded that these represent a minimal risk to human health, although assuming additivity, the mixture of PhACs could potentially present a hazard. On top of that, special concern have received the antibiotics owing to the promotion of antibiotic-bacterial resistance (ABR), and particularly, the maintenance and spread of antibiotic-resistant genes (ARG) among human pathogenic bacteria (Pärnänen et al., 2019; Y. Wu et al., 2016; Yang et al., 2019).

The wastewater treatment plants (WWTPs) were not designed to remove trace organic contaminants and only a fraction of each PhAC and their metabolites can be removed. The removal efficiency appears to be dependent on the specific compound and on the performance and type of wastewater technology used (Grandclément et al., 2017; Verlicchi et al., 2012). To overcome these problems, many papers have been published regarding the fate and removal efficiencies (REs) of PhACs in full-scale and pilot-scale WWTPs, with higher focus in the water line compared to the sludge line. Besides, few of them have attempted to relate the REs of PhACs to the changes of the operational parameters and other uncontrolled variables with the shifts in the structure and diversity of microbial communities of activated sludge processes (water line) and anaerobic digestion process (AD) (sludge line). Most of these studies are focused mainly in a conventional active sludge process (CAS) or membrane bioreactor system (MBR) in water line and conventional AD of sewage sludge in sludge line, but only a few of them have studied the REs of PhACs in advanced process such as integrated fixed film activated sludge (IFAS) systems (Grandclément et al., 2017; Krzeminski et al., 2019; Shreve and Brennan, 2019) and two-phase AD systems.

Therefore, is it important to find out the occurrence and fate of each PhAC in the whole process of a WWTP, focusing in the REs capacity of both, wastewater and sewage sludge treatment process (conventional *vs* advance), and understanding the most important operational parameter linked to the microbial community that can favored the RE of each PhAC.

2. Pharmaceutical consumption

To assess the amount of PhACs worldwide consumed is a difficult issue, and only a rough approximation has been estimated. About 4000 PhACs there are available for medical and veterinary purposes, resulting an annual production over 100,000 tons of pharmaceuticals (Boxall et al., 2012; Miarov et al., 2020). After United States of America, the EU ranks second for pharmaceutical sales with about 25% of the world sales for human consumption and 31% for veterinary consumption. In the EU there are around 3000 human active pharmaceuticals authorized and new drugs are being developed and produced each year, for instance, the numbers of new pharmaceuticals administrated per year has nearly double in the last decade (between 2005 and 2014) and 43 new PhACs were launched in 2015 only in the United States (Lockwood and Saidi, 2017; Miarov et al., 2020).

Furthermore, there is an increasing trend in the consumption of human pharmaceuticals due to growing world population, advanced research, global market availability and aging society. This trend has been driven in particular by the use of PhACs related to ageing and chronic diseases (antihypertensives, lipid-regulators, antidiabetics, and antidepressants) (Lockwood and Saidi, 2017), and also by the use of antimicrobials which increased substantially (35% increase between 2000 and 2010), in particular for the antibiotics carbapenems and polymyxins. This trend in the use of antibiotics occurred in developing countries, while in developed countries their overall use has recently slowed down. (Lockwood and Saidi, 2017; Van Boeckel et al., 2014).

Table I-1 shows the amount of 27 selected PhACs consumed in Spain from 2014 to 2017. The consumption is commonly expressed by Defined Daily Dose (DDD) unit per 1000 inhabitant per day (DHD), the value for DDD (mg active compound d^{-1}) is reported by the World Health Organization (WHO) Collaborating Center for Drugs Statistics (https://www.whocc.no/atc_ddd_index/) and the data for DHD were obtained by the Spanish Agency of Drugs and Healthcare Products (Agencia Española de Medicamentos y Productos Sanitarios, AEMPS). The DHD data account only the number of packages dispensed in pharmacy charged to the National Healthcare System. It does not include consumption that coming from private prescriptions, hospital, farm and over-the-counter drugs, except for the antibiotic group that include hospital and private prescriptions. The highest consumptions in terms of mass consumption (mg active compound 1000 inh^{-1} d^{-1}) correspond to the anti-inflammatory and/or analgesic pharmaceuticals (AIAPs)

acetaminophen, ibuprofen and naproxen, followed by the lipid-regulators gemfibrozil and fenofibrate, the psychiatric medicament carbamazepine, the diuretic furosemide and the antibiotics clarithromycin and sulfamethoxazole.

Table I-1. Amount of the 27 selected PhACs consumed in Spain from 2014 to 2017 given as DHD (Daily Dose Defined per 1000 inhabitant per day), DDD (Daily Dose Defined, mg active compound per day) and Mass consumption (mg active compound per 1000 inhabitant per day). *The corresponding years for paroxetine are 2011, 2012 and 2013.

Parameter	**DDD (mg active compound d^{-1})**	**DHD (DDD d^{-1} 1000 inh^{-1})**				**Mass consumption (mg d^{-1} 1000 inh^{-1})**
Year	**Actualized 2020**	**2014**	**2015**	**2016**	**2017**	**2016**
AIAPs		**58.03**	**55.40**	**56.37**		**102867**
Acetaminophen	3000	23.23	23.91	24.53	24.88	73588
Ibuprofen	1200	17.53	14.04	13.67		16404
Naproxen	1200	8.37	9.15	9.89		11868
Ketoprofen	150	2.69	2.76	3.01		452
Diclofenac	100	4.23	3.47	3.25		325
Codeine	100	1.61	1.73	1.71	1.73	171
Indomethacin	100	0.37	0.33	0.30		30
Propyphenazone	3000			0.01		30
Antibiotics		**1.21**	**1.25**	**1.23**	**1.17**	**1320**
Clarithromycin	750	0.786	0.831	0.801	0.745	601
Sulfamethoxazole	2000	0.279	0.281	0.284	0.288	569
Trimethoprim	400	0.090	0.091	0.092	0.093	37
Sulfamethazine	4000	0.001	0.001	0.001	0.001	4
Sulfadiazine	600	0.007	0.007	0.006	0.005	4
Ofloxacin	400	0.011	0.005	0.004	0.004	2
Beta-blocker		**8.27**	**8.22**	**7.98**	**7.66**	**722**
Atenolol	75	7.04	6.73	6.44	6.13	483
Propranolol	160	0.68	0.68	0.74	0.75	118
Metoprolol	150	0.44	0.69	0.68	0.66	102
Sotalol	160	0.11	0.11	0.12	0.12	19
Diuretics		**29.39**	**30.01**	**30.42**	**30.55**	**1045**
Furosemide	40	17.40	18.19	18.99	19.48	760
Hydrochlorothiazide	25	11.99	11.82	11.43	11.07	286
Lipid-regulators		**6.21**	**6.17**	**6.15**	**6.13**	**2645**
Gemfibrozil	1200	1.51	1.43	1.38	1.33	1651
Fenofibrate	200	4.58	4.64	4.68	4.71	936
Bezafibrate	600	0.12	0.11	0.10	0.09	58
Psychiatric medications		**31.46**	**31.61**	**32.16**		**1066**
Carbamazepine	1000	0.96	0.92	0.92		920
Paroxetine*	20	9.61*	10.03*	11*		220*
Diazepam	10	8.63	8.82	9.09	9.02	91
Lorazepam	2.5	21.87	21.87	22.15	22.11	55

3. European Union Legislation on PhACs in the aquatic environment

Table I-2 reports the main European Union (EU) legislation on medicinal products and the EU legislation concerning the protection of aquatic and soil environments in terms of priority substances and emerging contaminants as PhACs. The European Commission

Directives 2001/83/EC on human medicines and 2001/82/EC on veterinary medicines are the main means for ensuring the quality, efficacy and safety of drugs for human and animals use, and their safety for the environment with the establishment of the environmental risk assessment (ERA). For marketing authorization, the human and veterinary medicines should include the ERA study, where, the undesirable effects on the environment produced for a human medicine is not a criterion for refusal from the market, whereas the environmental impact for veterinary medicinal products can be a basis for refusal of market authorization.

The Directives concerning the protection of aquatic environments is mainly represented by the Water Framework Directive 2000/60/EC (WFD), the Ground Water Directive 2006/118/EC (GWD) and the Directive on Environmental Quality Standards 2008/105/EC (EQSD). The WFD and GWD were created to reach a better ecological and chemical status of the European water bodies. The Directive 2008/105/EC established the EU List of Priority Substances, 33 pollutants for surface waters with the values of the Environmental Quality Standards (EQS). The inclusion in the EU List of Priority Substances implies the necessity for monitoring the water bodies, sediment and biota with the definition of the corresponding EQS. In 31 of January 2012, the EU Commission proposed to add 15 new pollutants to the List (COM (2011) 876 final, 2011), where 1 PhAC and 2 hormones (17b-estradiol (E2), 17a-ethinylestradiol (EE2) and diclofenac were included for the first time among these 15 chemicals. In the following directive (Directive 2013/39/EU) twelve chemicals were added to the List of Priority Substances, but no PhACs was included among them, notwithstanding the occurrence and environmental effects that have been documented for more than 20 years before in many European countries (Verlicchi et al., 2012).

It was not until 2015 when the European Commission decided to include 4 PhACs (the analgesic diclofenac and the antibiotics azithromycin, clarithromycin and erythromycin) and 3 hormones (estrone (E1), E2 and EE2) in the "Watch List" of the EQSD (Commission Implementing Decision (EU) 2015/495) for monitoring and to discuss the future limits of discharge of these compounds. This Decision aimed to generate high-quality data on their concentration to address the environmental risk posed by those substances. During 2017 the Commission analyzed the data and concluded that sufficient high-quality monitoring data are available for the four substances, including the pharmaceutical diclofenac. Therefore, those substances have been removed from the new

"Watch List" of the EQSD (Commission implementing decision EU 2018/840**);** the same occurred with the macrolide antibiotics clarithromycin and erythromycin but those were no removed because the macrolide azithromycin need additional high-quality data and should be controlled together as a group because substances with the same mode of action could have additive effects. On the other hand, the antibiotics amoxicillin and ciprofloxacin were identified as suitable candidates and were included in the new Watch List; this is consistent with the European One Health Action Plan against Antimicrobial Resistance (COM(2017) 339, 2017).

Several States (The Netherlands, Sweden, Switzerland and United Kingdom) have expressed concerns and taken action to reduce the growing presence of PhACs in the environment (COM(2019) 128, 2019). For instance, the Swiss Federal Office for the Environment (FOEN) is a pioneer in creating a legal framework for PhACs in sewage wastewater effluents (FOEN, 2017). The aim is that each canton in Switzerland must remove over 80% the concentration of 6 compounds from a list of 10 PhACs and 2 industrial chemicals in the whole wastewater treatment process included a final step with advanced treatment process (ozone or activated carbon) (Alvarino et al., 2018a; Bourgin et al., 2018). The PhACs selected for the list were: the psychiatric medications amisulpride, carbamazepine, citalopram and venlafaxine, the antibiotic clarithromycin, the anti-inflammatory diclofenac, the diuretic hydrochlorothiazide, the beta-blocker metoprolol, and the antihypertensives candesartan and irbesartan.

The basic EU legislation concerning the sewage sludge management is the Directive 86/278/EEC on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. This text set out rules for use sewage sludge as a fertilizer and set out limits on the concentration of heavy metals in the soil, sewage sludge, as well as, the maximum annual quantity of heavy metals that may be accumulated into the soil; another main EU legislation related to sewage sludge is the Directive 91/271/EEC, where it encourages the reuse and efficient management of the sewage sludge. Currently, no regulations in the EU legislation have been set for the presence of PhACs in the sewage sludge (Christodoulou and Stamatelatou, 2016; Martín-Pozo et al., 2019; Thomaidi et al., 2016), despite many organic micro-contaminants and PhACs have been found in digested sewage sludge, biosolids and agricultural soils for several years (Bourdat-Deschamps et al., 2017; Ekpeghere et al., 2017; Mailler et al., 2016; Phan et al.,

2018; Samaras et al., 2014; Thomaidi et al., 2016; Tien et al., 2017; Urra et al., 2019; Verlicchi and Zambello, 2015).

Table I-2. European Union legislation in terms of medicinal substance and organic micropollutants in the environment mainly in the aquatic and agricultural soil environment.

Directives	**EU Legislation on medicinal products**
Directive 2001/83/EC ***In force**	Directives related to human medicine where it established the environmental risk assessment of human drugs.
Directive 2001/82/EC ***In force**	Directives related to veterinary medicine where it established the environmental risk assessment of veterinary drugs.
Directive 2010/84/EU ***In force**	Directives related to human medicine on pharmacovigilance.
Regulation (EU) 2019/6 of the EU 11 December 2018 on veterinary medicinal products and human medicine. ***In force**	An Environmental risk assessment (ERA) is now mandatory for all applications and marketing authorization for human and veterinary medicinal products.
Directives	**EU Legislation concerning the protection of aquatic environments in terms of priority substances.**
Water Framework Directive 2000/60/EC (WFD) ***In force**	Define "Strategies against pollution of water".
Decision 2455/2001/EC	First List of priority substance with 33 substances identified as major concern for European waters, within 11 substances identified as priority hazardous.
Ground Water Directive 2006/118/EC (GWD) ***In force**	Directive for protection of groundwater against pollution and deterioration.
Directive on Environmental Quality Standards 2008/105/EC (EQSD) ***In force**	Directive on Environmental Quality Standards (EQSs) where it was established the EU List of Priority Substances of 33 pollutants.
Directive 2013/39/EU ***In force**	12 chemicals added to the WFD List of Priority Substances.
Commission implementing decision (EU) 2015/495 ***No longer in force**	The "watch-list" of substances for monitoring in the field of water policy pursuant to Directive 2008/105/EC was published and include four PhACs (the analgesic diclofenac and the antibiotics azithromycin, clarithromycin and erythromycin) and three hormones (estrone (E1), 17b-estradiol (E2) and 17a-ethinylestradiol (EE2)).

Commission implementing decision (EU) 2018/840 ***In force**	The commission established a "new Watch List" of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495.

Directives	**EU Legislation concerning the sewage sludge management in terms of priority substances**.
Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. *** In force**	Directive to set rules for use sewage sludge as a fertilizer, it sets limits on the concentration of heavy metals in the soil, sewage sludge and maximum annual quantities of heavy metals that may be added to the soil. No mention of organic micropollutants as PhACs has been made.
Council Directive 91/271/EEC of 21 May 1991 concerning urban waste-water treatment *** In force**	The objective of the Directive is to protect the environment from the adverse effects of the wastewater discharges and encourage and efficient management of the sludge.

4. Physico-chemical properties of pharmaceutically active compounds

Table I-3 shows the main physicochemical properties of the selected PhACs studied in this Thesis. Most PhACs are small (MW<1000 Da) organic molecules that can be distributed between different environmental compartments (e.g. groundwater and surface water, sediment or soils) according to its physicochemical properties such as, volatility (Henry's Law), solubility (S_w 25ºC), acidity (pK_a), lipophilicity ($LogK_{ow}$) and sorption potential (Kd). The pharmaceuticals are designed to meet a wide range of medical needs, and the classification is complex. For simplicity, they are classified following the Anatomical Therapeutic Chemical Classification System (ATC system) (WHO Collaborating Centre for Drugs Statistics Methodology – WHOCC). The selected pharmaceutical groups showed in Table I-3 (AIAPs, antibiotics, beta-blockers, diuretics, lipid-regulators and psychiatric medications) correspond to the most studied group in literature due to the higher consumption and presence in the environment (Luo et al., 2014; Tran et al., 2018; Verlicchi et al., 2012). Even though each PhACs from the same group has a similar therapeutic application, they show different acidity, solubility, lipophilicity and sorption potential that influence their fate in the WWTP process and environment.

Henry's law coefficient shows the tendency of a compound to volatilize from the liquid phase. Ternes and Joss (2015) observed that if Henry's coefficient is above 0.003

Molar.atm^{-1} a significant amount of a compound could be stripped in a bioreactor with fine bubble aeration. Therefore, due to the lower Henry's constant for the selected PhACs, it can be concluded that the removal by stripping process is not relevant for the PhACs during the WWTPs.

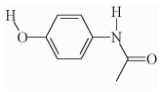
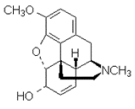
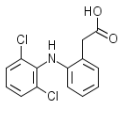
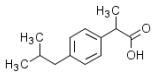
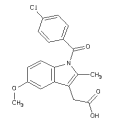
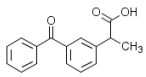
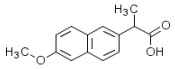
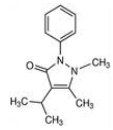
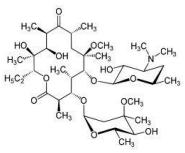
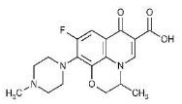
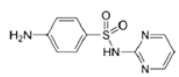
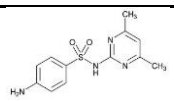
The acidity (pK_a) indicated the ionization degree of the compound at a known pH. If the pK_a value is higher than 7 the compound will be charge positively in acidic condition and vice versa. The pH value range normally between 4 and 8 in the environment, between 6 and 7.5 in the sewage sludge and between 7 and 8 in the activated sludge (Tchobanoglous et al., 2003). There is high variability in the acidity and charge at pH 7 of the selected PhACs.

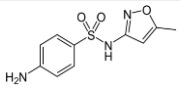
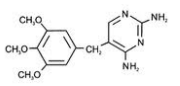
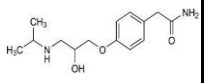
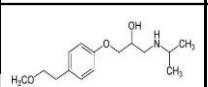
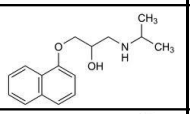
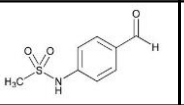
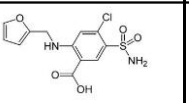
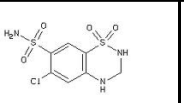
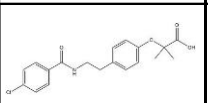
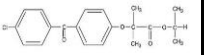
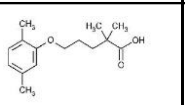
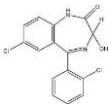
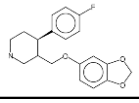
The lipophilicity indicates the capacity of the compound to be repelled to a mass of water or attracted to the particle matter. There are different experimental coefficients to evaluate this tendency, the most common parameters used is the octanol-water partition coefficient (K_{ow}) and the pH-dependent octanol-water distribution coefficient (D_{ow}). As known, the PhACs are multifunctional organic molecules that can be ionizable, acidic or basic, which can exist as negative, positive or neutral charged, where the partitioning depends on pH and pK_a, therefore Cunningham (2008) reported that the pH-dependent D_{ow} coefficient is more appropriate for understanding the distribution of the PhACs in different environmental matrixes. Nevertheless, the best parameter to analyze the capacity of the compound to remain in water phase or solid phase (organic matter, biosolid, activated sludge) is the sorption potential indicated by the experimental solid-water distribution coefficient (K_d), which is the ratio of the quantity found in the solid phase to the amount found in the water phase (Carballa et al., 2008; Martín et al., 2012b; Radjenović et al., 2009; Ternes et al., 2004; Tran et al., 2018).

A significant variation in K_d value of each compound has been reported among different studies (see the high range in Table I-3), it can be due to the differences in methodology, difference type of matrix (influent wastewater, activated sludge, raw or digested sewage sludge, etc.) and difference operational condition of the WWTPs, such as pH, solid retention time (SRT), temperature, and reactor configuration (Tran et al., 2018). Furthermore, sorption of PhACs onto activated or sewage sludge is strictly dependent on their physicochemical properties describe before and molecular structure. To date, a simple rule has been widely accepted, if the compound has log K_{ow} > 4.0 or log D_{ow} > 3.0, it potentially tend to adsorb onto the sludge or particulate phase, having a high sorption potential (K_d > 1000 L.Kg^{-1}), while compounds with log K_{ow} < 2.5 or log D_{ow} <

1.0 often tend to have low sorption potential (K_d < 300 $L.Kg^{-1}$) and log K_{ow} between 2.5 and 4 or log D_{ow} between 1.0 or 3.0 have usually medium sorption potential (K_d between 300 and 1000 $L.Kg^{-1}$) (Luo et al., 2014; Tran et al., 2018). However, these two coefficients (K_{ow} and D_{ow}) not always follow a clear correlation with the sorption potential (K_d) (Tran et al., 2018). For example, of the 27 PhACs selected in this Thesis, 9 compounds (diclofenac, ibuprofen, indomethacin, ketoprofen, naproxen, ofloxacin, atenolol, bezafibrate and gemfibrozil) do not follow this rule. Therefore, to estimate the sorption potential and range of partitioning between the water phase and particle phase, the best would be calculated the Kd coefficient for each compound and type of matrix.

Table I-3. Physico-chemical properties of the selected pharmaceuticals. Data were obtained from the review papers of Tran et al. (2018), Verlicchi et al. 2012 and Verlicchi and Zambello (2015).* the Kd's data represent the range reported in the literature for all types of sewage sludge.

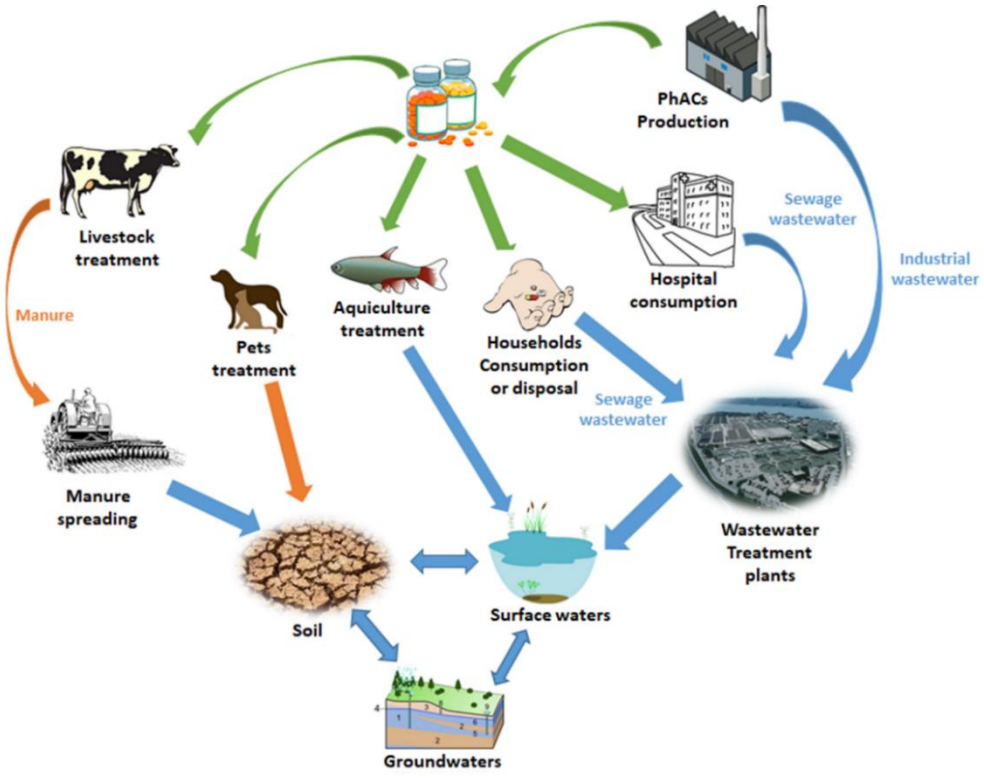
	Pharmaceutical	Molecular structure	Henty's Law constant (Molar.atm^{-1})	pK_a	S_w 25°C (mg l^{-1})	Charge at pH 7	*Kd (L.kg^{-1}) of all type's sludge	logK_{ow}	References
Analgesics/Anti-inflammatories (AIAPs)	Acetaminophen		6,42E-13	9,38	3.035x10^4	Neutral	0 - 1160	0,46	Jones et al., 2002
	CAS # 103-90-2							1,59	Kinney et al., 2008
	MW: 151 g.mol^{-1}							0.27-0.46	Verlicchi et al., 2012
								1.18-1.53	Xia et al., 2005
	Codeine		7,58E-14	8,21	1.21x10^4	Positive	14	1,28	Subedi et al., 2014
	CAS # 76-57-3								
	MW: 299 g.mol^{-1}								
	Diclofenac		4,73E-12	4,15	4,52	Negative	1.9 - 501	2,16	Hyland et al., 2012
	CAS # 15307-86-5							0,7	Jones et al., 2002
	MW: 296 g.mol^{-1}							4-4.7	Salgado et al., 2012
								4,6	Ternes et al., 2004
								4.51-4.8	Verlicchi et al., 2012
	Ibuprofen		1,50E-07	4,51	41,05	Negative	6 - 360	3,5	Jones et al., 2002
	CAS # 15687-27-1							3,97	Khan and Onghert, 2002
	MW: 206 g.mol^{-1}							4,5	Verlicchi et al., 2012
								0.82-3.4	Xia et al., 2005
	Indomethacin		3,13E-14	4,5	3,114	Negative	2.5 - 3.2	4,27	Verlicchi et al., 2012
	CAS # 53-86-1								
	Ketoprofen		2,12E-11	4,45	120,4	Negative	16 - 226	3-3.2	Salgado et al., 2012
	CAS # 22071-15-4								
	MW: 254 g.mol^{-1}							3,16	Verlicchi et al., 2012
	Naproxen		3,39E-10	4,19	144,9	Negative	10 - 145	3,18	Jones et al., 2002;
	CAS # 22204-53-1								
	MW: 230 g.mol^{-1}								
	Propyphenazone		1,84E-09		668,2	Neutral		1,96	Verlicchi et al., 2012
	CAS # 479-92-5								
	MW: 230 g.mol^{-1}								
Antibiotics	Clarithromycin		1,73E-29	8,99	0,342	Positive	260 - 1200	3,18	EPI Suite
	CAS # 81103-11-9								
	MW: 248 g.mol^{-1}								
	Ofloxacin		4,98E-20	5,97	2.826x10^4	Neut./Neg.	137 - 22100	0,35	Verlicchi et al., 2012
	CAS # 82419-36-1								
	MW: 361 g.mol^{-1}								
	Sulfadiazine		1,58E-10	pK$_1$= 6.36	2.814x10^4	Neut./Neg.	4.53 - 52.6	-0,09	Gao et al., 2012
	CAS # 68-35-9								
	MW: 250 g.mol^{-1}			pK$_2$= 2.1					
	Sulfamethazine		3,05E-13	2,65	1.124 10^4	Neut./Neg.	13.2 - 177	0,89	Gao et al., 2012
	CAS # 57-68-1								
	MW: 278 g.mol^{-1}								

	Pharmaceutical	Molecular structure	Henty's Law constant (Molar.atm^{-1})	pK_a	S_w 25°C (mg l^{-1})	Charge at pH 7	*Kd (L/kg) of all type's sludge	logK_{ow}	References
Antibiotics	Sulfamethoxazole		6,42E-13	6,16	3942	Neut./Neg.	3.2 - 98.2	0,89	Gao et al., 2012
	CAS # 723-46-6								
	MW: 253 g.mol^{-1}							0,5	Verlicchi et al., 2012
	Trimethoprim		2,39E-14	7,2	2334	Pos./Neut	25.4 - 427	0,91	Kinney et al., 2008
	CAS # 738-70-5							0,73	Subedi et al., 2014
	MW: 290 g.mol^{-1}								
Beta-Blokers	Atenolol		5,70E-13	9,6	685,2	Positive	5.9 - 1900	0,16	EPI Suite
	CAS # 29133-68-7								
	MW: 266 g.mol^{-1}								
	Metoprolol		1,40E-13	9,6	4777	Positive	18 - 137.8	1,88	EPI Suite
	CAS # 37350-58-6								
	MW: 2267 g.mol^{-1}								
	Propranolol		7,98E-13	9,42	228	Positive	269 - 641	3,48	EPI Suite
	CAS # 525-66-6							2,6	Subedi et al., 2015
	MW: 250 g.mol^{-1}								
	Sotalol		2,49E-14	pK_1=8.2	5513	positive	18 - 740	0,24	EPI Suite
	CAS # 3930-20-9			pK_2=9.8					
	MW: 259 g.mol^{-1}								
Diuretics	Furosemide		3,94E-16	3,9	149,3	Negative	110 - 127	2,03	EPI Suite
	CAS # 54-31-9								
	MW: 331 g.mol^{-1}								
	Hydrochlorothiazide		4,39E-12	7,9	1292	Negative	20 - 92	-0,07	EPI Suite
	CAS # 58-93-5								
	MW: 298 g.mol^{-1}								
Lipid-regulators	Bezafibrate		2,12E-15	3,6	1,224	Negative	28 - 119	4,25	EPI Suite
	CAS # 41859-67-0								
	MW: 362 g.mol^{-1}								
	Fenofibrate		4,46E-09	4,5	0,1957	Neutral	-	5,19	EPI Suite
	CAS # 49562-28-9								
	MW: 361 g.mol^{-1}								
	Gemfibrozil		1,19E-08	4,42	4,964	Negative	19.3 - 129	4,77	EPI Suite
	CAS # 25812-30-0								
	MW: 250 g.mol^{-1}								
Psychiatric Medications	Carbamazepine		1,08E-10	15,6	17,66	Neutral	8 - 330	2,45	Kinney et al., 2006
	CAS # 298-46-4							2,25	Subedi et al., 2015
	MW: 236 g.mol^{-1}								
	Diazepam		3,64E-09	2,92	58,78	Neutral	21 - 161	2,82	EPI Suite
	CAS # 439-14-5							2,7	Subedi et al., 2015
	MW: 285g.mol^{-1}								
	Lorazepam		4,10E-10	13	83,87	Neutral	958	2,39	EPI Suite
	CAS # 846-49-1							3,95	Subedi et al., 2015
	MW: 250 g.mol-1								
	Paroxetine		1,78E-12	9	35,27	Positive	8600 - 35000	3,95	EPI Suite
	CAS # 61869-08-7								
	MW: 329 g.mol^{-1}								

5. Sources and pathways of PhACs in the environment

Figure I-1 shows the major sources and pathways of PhACs that can enter into the environment. The sewage WWTP effluents are considered the main emission pathway of PhACs into the surface waters, which receive the PhACs excreted from household and hospital consumers. Many studies show that, despite metabolism, the active substances are excreted mostly between 30 and 90% of the orally administrated dose (Al Aukidy et al., 2012; Jjemba, 2006; Lienert et al., 2007; Lockwood and Saidi, 2017; Verlicchi et al., 2014). The second most-often source for PhACs is the veterinarian drugs administered to livestock where a substantial amount of active compound can be excreted via animal urines and faeces. These PhACs can reach surface waters, groundwater and soil via surface run-off and manure land application (aus der Beek et al., 2016; Boxall et al., 2012; Tien et al., 2017). Other important emission sources and pathways are the aquaculture facilities, drugs manufacturing plants, veterinarian drugs for pets and irrigation with reclaimed water and sewage biosolids agricultural land application (Thomaidi et al., 2016; Wu et al., 2015).

Figure I-1. Main sources and pathways of PhACs release into the environment (Boxall et al., 2012; Kümmerer, 2009).



6. Occurrence and fate of PhACs in wastewaters and sewage sludge of municipal WWTPs

During the last two decades, many studies have been conducted to find the occurrence, face, mass balance and removal efficiency of many PhACs in full and pilot-scale WWTPs around the world. A full literature review is set out in order to collect data of the 27 selected PhACs from different WWTPs operating at different biological process in water line such as CAS, MBR, IFAS systems and AD in sludge line. The objective is to provide a snapshot of the occurrence and fate of the difference biological processes reported in the literature for the selected PhACs.

6.1. Occurrence of PhACs in the wastewater influent and secondary effluent of municipal WWTPs

Literature range concentration values for the selected 27 PhACs in the raw influent and secondary effluent of municipal WWTPs are shown in Figure I-2 and Figure I-3, respectively. The compounds are grouped in alphabetic order in their therapeutic classes, with the mean value shown in brackets in the X-axis after the name of each compound. The numbers of paper and references revised for each compound are shown in Table I-S1. A total of 70 papers have been revised, where 60 papers reported data from full CAS-WWTPs, 7 paper from full-scale CAS-WWTP and pilot-scale MBR plants, 1 paper from 4 different CAS-WWTP and one full-scale MBR-WWTP (Park et al., 2017) and 1 paper reported data from 6 advance full-scale IFAS-WWTPs (Shreve and Brennan, 2019). The majority of the studies were done in Europe (42 papers) followed by Asia (14), North America (11), Australia (2) and South America (1).

The most commonly investigated compounds were the anti-inflammatories ibuprofen, diclofenac, naproxen and ketoprofen, the antibiotics sulfamethoxazole and trimethoprim and the psychiatry medicament carbamazepine, while concentration data were not available for fenofibrate in the influent, hydrochlorothiazide and paroxetine in the effluent.

Referring to Figure I-2 the highest average influent concentration values correspond for the AIAPs acetaminophen (52.41 µg L^{-1}), ibuprofen (17.25 µg L^{-1}), naproxen (5.38 µg L^{-1}) and codeine (5.01 µg L^{-1}), with the highest absolute influent concentration for acetaminophen (400 µg L^{-1}) and ibuprofen (192 µg L^{-1}), indicating that these over-the-counter drugs are highly consumed. The following drugs with higher mean concentration are the diuretic hydrochlorothiazide (3.12 µg L^{-1}), the beta-blocker atenolol (2.89 µg L^{-}

[1]), the antibiotic ofloxacin (2.32 µg L^{-1}), the lipid-regulators bezafibrate (1.92 µg L^{-1}) and psychiatry medicament carbamazepine (1.50 µg L^{-1}), the mean concentration of the rest are below 1.50 µg L^{-1}, there are another compounds with higher mean concentration such as diazepam (7.33 µg L^{-1}), but high variability and low number of data (6) was reported. No data were found for fenofibrate and very low concentration and data were provided for propyphenazone, sulfamethazine, lorazepam and paroxetine.

Figure I-2. Concentration of the 27 selected PhACs measured in the raw influent municipal WWTPs reported in the bibliography (Table I-S1). The corresponding mean concentration values are in brackets (ng L^{-1}).

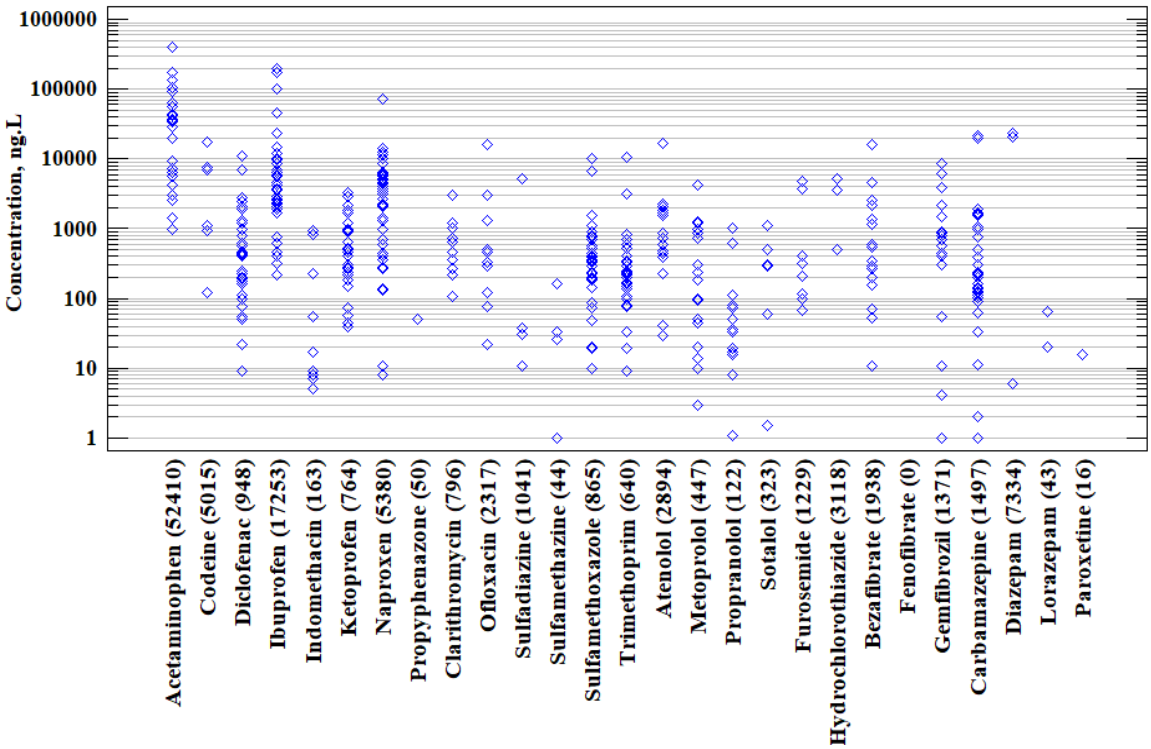


Figure I-3 shows the concentration of the 27 selected PhACs detected in the secondary effluent of the WWTPs reviewed in this study, together with the minimum predicted no-effect concentration (PNEC) values (marked with a red line) found in the literature (Table I-4) (Al Aukidy et al., 2012; Orias and Perrodin, 2013; Papageorgiou et al., 2016; Tran et al., 2018). According to European Commission (2003), the measured environment concentration of PhACs higher than the PNEC could represent a risk for aquatic ecosystems; in this sense, the PhACs with secondary effluent concentration values

higher than the PNEC could damage the aquatic life, especially in dry effluent-site rivers with low dilution factor (Kasprzyk-Hordern et al., 2009; Verlicchi et al., 2012).

The highest average secondary effluent concentration values correspond for the psychiatry medicaments diazepam (4036 ng L^{-1}) and carbamazepine (1442 ng L^{-1}), as well as, the AIAPs ibuprofen (1619 ng L^{-1}), codeine (1131 ng L^{-1}), diclofenac (828 ng L^{-1}) and acetaminophen (741 ng L^{-1}). Contradictory data have been found for diazepam with 7 values below 20 ng L^{-1} and two values very high (>15 µg L^{-1}). Otherwise, very low concentration and data were found for propyphenazone, fenofibrate, sulfadiazine, sulfamethazine, and paroxetine.

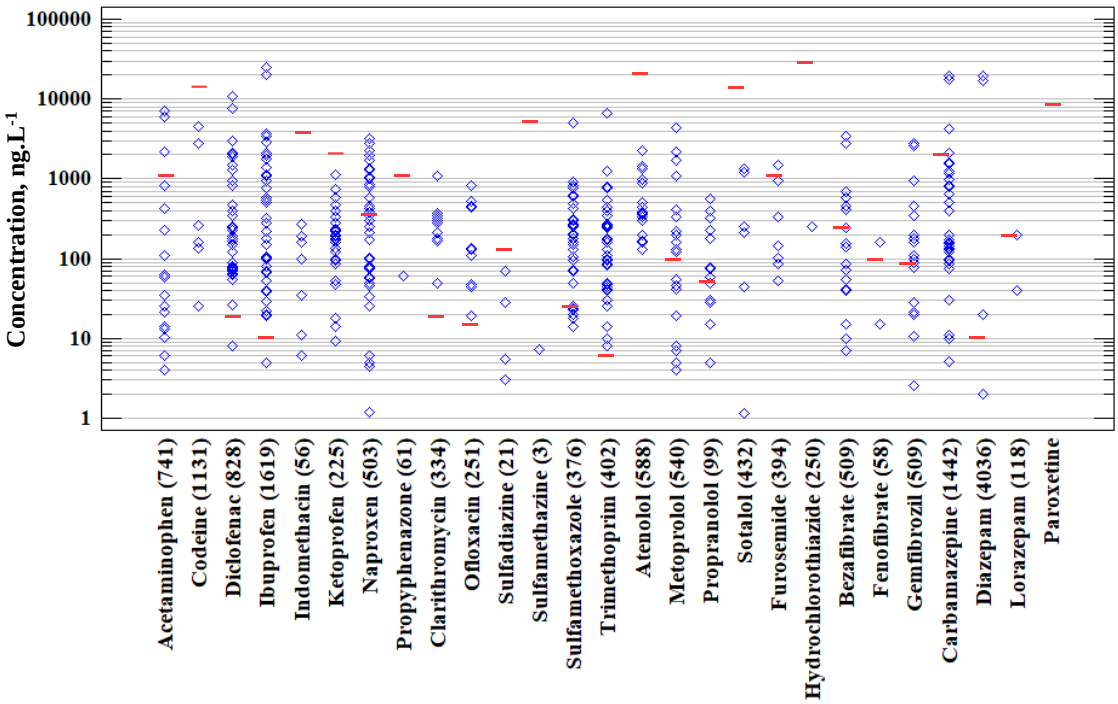
To identify the most dangerous compounds in the environment, a rough estimation of the environmental risk posed by these PhACs in secondary effluent was done by means of the risk quotient (RQ), which is the ratio between the average PhACs concentration in the secondary effluent and its corresponding PNEC. A simple criterion was applied according to Hernando et al. (2006) and Verlicchi et al. (2012): RQ<0.1 low risk to aquatic organism; 0.1≤RQ≥1 medium risk; and RQ≥1 high risk. The RQ values for the 27 selected PhACs are shown in Table I-4, where 12 compounds pose a high risk (diazepam, ibuprofen, trimethoprim, diclofenac, clarithromycin, ofloxacin, sulfamethoxazole, gemfibrozil, metoprolol, bezafibrate, propranolol, and naproxen), and 7 compounds pose medium risk (acetaminophen, carbamazepine, lorazepam, fenofibrate, furosemide, sulfadiazine and ketoprofen).

Table I-4. Minimum predict not-effect concentration (PNEC) found in the bibliography and risk quotient (RQ) ratio calculated between the average concentration of PhACs in secondary effluent and PNEC.

Groups	Pharmaceutical compounds	Minimum PNEC, ng/L	References	Risk Quotient (RQ)
AIAPs	Acetaminophen	1000	Tran et al., 2018	0,74
	Codeine	16000	Verlicchi et al., 2012	0,07
	Diclofenac	20	Orias and Perrodin, 2013	**41**
	Ibuprofen	10	Tran et al., 2018	**162**
	Indomethacin	3900	Tran et al., 2018	0,01
	Ketoprofen	2000	Orias and Perrodin, 2013	0,11
	Naproxen	330	Tran et al., 2018	**1,52**
	Propyphenazone	1000	Verlicchi et al., 2012	0,06
Antibiotics	**Clarithromycin**	20	Verlicchi et al., 2012	**17**
	Ofloxacin	16	Verlicchi et al., 2012	**16**
	Sulfadiazine	135	Verlicchi et al., 2012	0,16

	Sulfamethazine	5000	Tran et al., 2018	0,00
	Sulfamethoxazole	27	Tran et al., 2018	**14**
	Trimethoprim	5.8	Orias and Perrodin, 2013	**69**
Beta-blocker	Atenolol	20000	Orias and Perrodin, 2013	0,03
	Metoprolol	100	Orias and Perrodin, 2013	**5,40**
	Propranolol	50	Orias and Perrodin, 2013	**1,99**
	Sotalol	13000	Orias and Perrodin, 2013	0,03
Diuretics	Furosemide	1000	Orias and Perrodin, 2013	0,39
	Hydrochlorothiazide	317000	Orias and Perrodin, 2013	0,00
Lipid-regulators	**Bezafibrate**	230	Tran et al., 2018	**2,21**
	Fenofibrate	100	Verlicchi et al., 2012	0,58
	Gemfibrozil	78	Tran et al., 2018	**6,53**
Psychiatric medications	Carbamazepine	2000	Orias and Perrodin, 2013	0,72
	Diazepam	10	Orias and Perrodin, 2013	**404**
	Lorazepam	200	Orias and Perrodin, 2013	0,59
	Paroxetine	8800	Orias and Perrodin, 2013	0,00

Figure I-3. Concentration of the selected PhACs measured in the secondary effluent of the WWTPs reported in the bibliography (Table I-S1). The corresponding mean concentration values are in brackets (ng L^{-1}). The red lines represent the minimum PNEC value reported in bibliography (Table I-4**).**



6.2. Occurrence and fate of PhACs in the raw sewage sludge and digested sludge of WWTPs

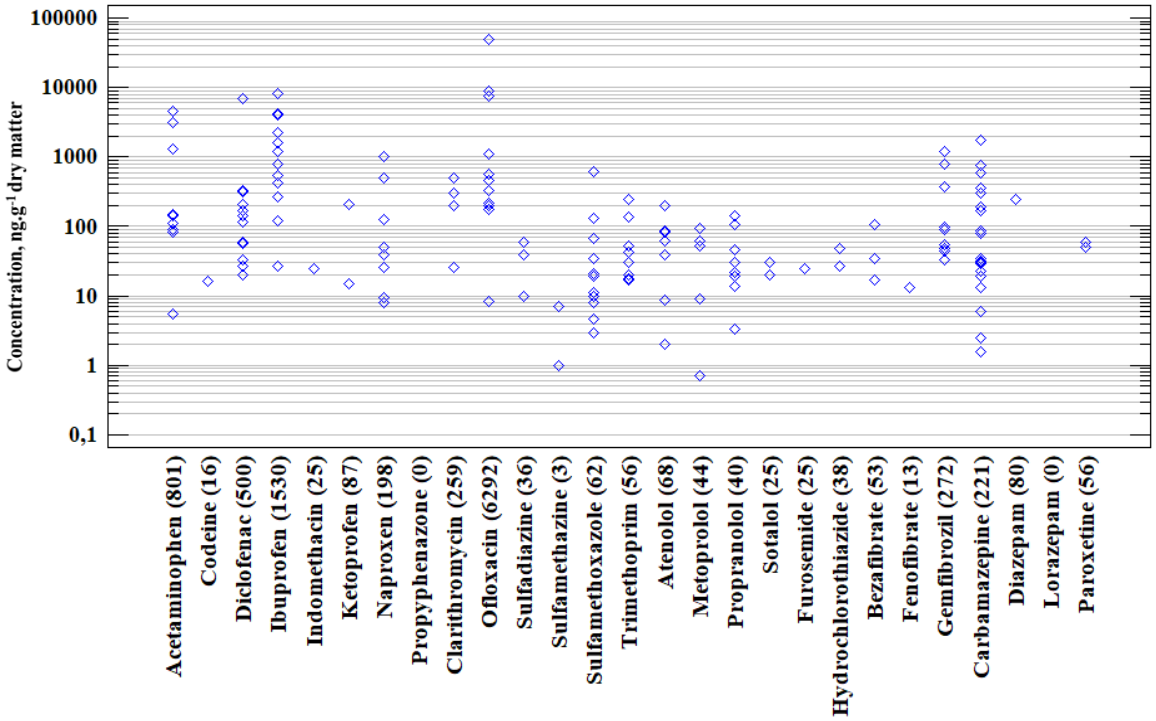
Sewage sludge is a by-product semi-solid generated during the primary treatment (physical and/or chemical process) and secondary biological treatment of a municipal WWTPs. Different types of sludge are produced in a WWTP, such as primary sludge coming from the primary sedimentation, secondary sludge obtained from the activated sludge sedimentation, mixed sludge obtained from the mixing of both sludges, thickened sludge coming from the thickening of primary and secondary sludge and stabilized or conditioned sludge obtained after anaerobic or aerobic digestion process.

Due to the high content of valuable organic matter and nutrients such as nitrogen and phosphorous, the conditioned sewage sludge has been used in agricultural soils over a long time. Approximately 40% of sludge produced in the EU is used in agriculture as amendment organic biosolids and over 12% is used to produce compost (Ivanová et al., 2018). Although the application of sewage sludge can be beneficial to soil, also can increase the concentration of heavy metals, pathogens and organic micro-contaminants such as PhACs. Apart from heavy metals and pathogens, not regulation have been set for the presence of PhACs in the sewage sludge (see Table I-2 and section 3) notwithstanding that the presence of these pollutants in the soil may have several impacts on the organisms, in the biodiversity of soil and aquatic ecosystems, and can be accumulated in plants (Bourdat-Deschamps et al., 2017; Prosser and Sibley, 2015; Verlicchi and Zambello, 2015; Wu et al., 2015).

Figure I-4 shows the concentration reported in the bibliography (Table I-S2) for the 27 selected PhACs in the raw sewage sludge (primary, secondary, mixed or thickened sludge) normally used as influent of the AD process in a full WWTPs, and Figure I-5 shows the concentration of these PhACs measured in the digested sludge of the WWTPs reported in the bibliography (Table I-S2). The corresponding mean concentration values are in brackets (ng g^{-1} dry matter, DM). In general, data on the occurrence and fate of PhACs in sewage sludge are scarce compared to the data and studies found in the water line of the WWTPs due to the demanding effort needed in the analysis of this difficult matrix. A total of 23 papers have been revised where the majority of the studies were done in Europe (14 papers) followed by Asia (5), North America (2) and Australia (2).

The highest average raw sewage sludge concentration values correspond for the antibiotic ofloxacin (6299 ng g^{-1} DM), the AIAPs ibuprofen (1530 ng g^{-1} DM), acetaminophen (801 ng g^{-1} DM) and diclofenac (500 ng g^{-1} DM), with the highest absolute influent concentration for ofloxacin (50 µg g^{-1} DM), ibuprofen (8125 ng g^{-1} DM), and diclofenac (7020 ng g^{-1} DM). No data were found for propyphenazone and lorazepam and very low concentration and data were provided for codeine, fenofibrate and sulfamethazine.

Figure I-4. Concentration of the selected PhACs measured in the raw sewage sludge of the WWTPs reported in the bibliography (Table I-S2). The corresponding mean concentration values are in brackets after the compound name (ng g^{-1} dry matter).



The PhACs concentrations data of Figure I-5 were obtained from the digested sludge effluent of 19 municipal anaerobic digesters, 2 pilot-scale AD plants and 1 lab-scale AD plant. The minimum PNEC values were calculated for the digested sludge (Table I-5) and marked with a red line in the Figure I-5 for comparation.

The highest average digested sludge effluent concentration value corresponds by far for ofloxacin (22203 ng g^{-1} DM) followed by ibuprofen (1524 ng g^{-1} DM), carbamazepine (211 ng g^{-1} DM) and sulfamethazine (184 ng g^{-1} DM) with the highest absolute concentration value for ofloxacin (80 µg g^{-1} DM) and ibuprofen (8.1 µg g^{-1} DM).

On the other hand, very low concentration and data were found for lorazepam, sulfamethoxazole, sulfadiazine, sulfadiazine and propyphenazone.

To identify the compounds most susceptive to damage the environment, a rough estimation of the RQ ratio have been calculated (Table I-5) for the digested sludge. It is the ratio between the means PhACs concentration in the digested sludge and its corresponding PNEC calculated for the sludge. Due to the lack of data regarding to experimental toxicity for the effects of PhACs on terrestrial organisms, the $PNEC_{sludge}$ values has been calculated according to the following equation (European Commission, 2003; Martín et al., 2012b; Thomaidi et al., 2016; Verlicchi and Zambello, 2015):

$$\mathrm{PNEC}_{sludge} = PNEC_{water} \times K_d$$

Where Kd is the solid–water partition coefficient which takes into account the mechanisms of absorption and adsorption (Table I-3) and $PNEC_{water}$ is the minimum value for aquatic organism shown in Table I-4. The Kd of each compound depend of the different kind of matrix and physicochemical conditions, many authors have measured it in different type of sludge and soil. To obtained the worst-case scenario the minimum Kd and minimum $PNEC_{water}$ value have been taken from the bibliography for each compound in digested sludge. Following the criterion for RQ applied in the previous point, a high environmental risk could be posed by 12 compounds (acetaminophen, diclofenac, ibuprofen, naproxen, clarithromycin, ofloxacin, sulfamethoxazole, trimethoprim, metoprolol, bezafibrate and carbamazepine), and medium risk could be for 4 compounds (indomethacin, ketoprofen, propranolol and furosemide).

Figure I-5. Concentration of the selected PhACs measured in the digested sludge of the WWTPs reported in the bibliography (Table I-S2). The corresponding mean concentration values are in brackets after the name (ng g^{-1} dry matter). The red lines represent the minimum PNEC values calculated for digested sludge (Table I-4).

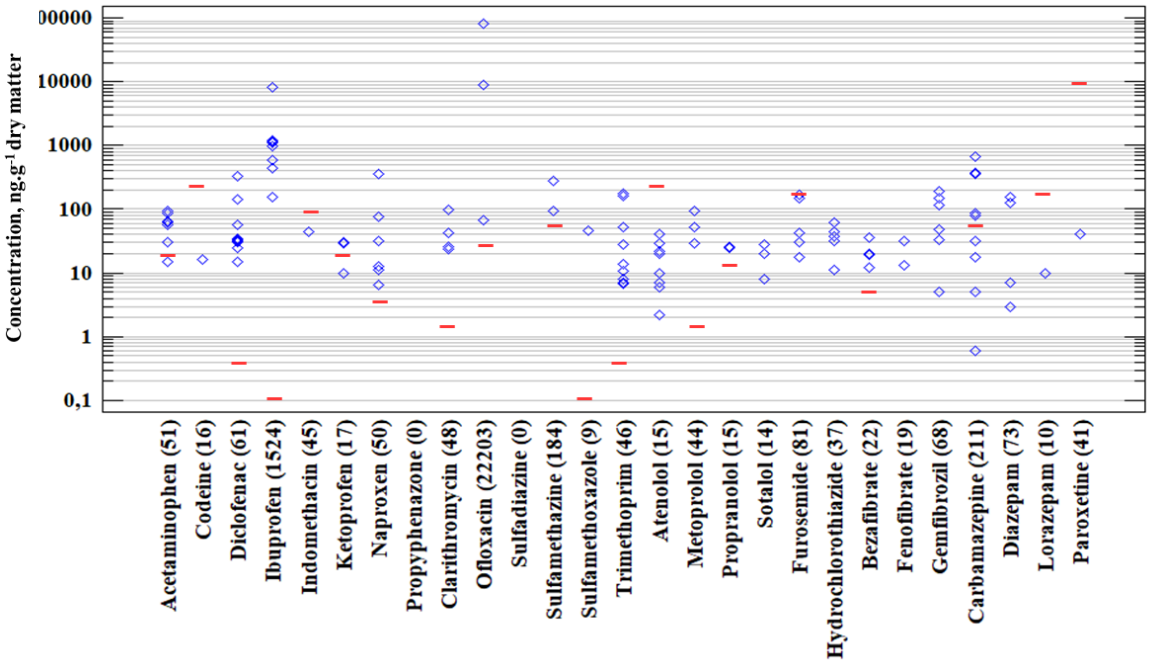


Table I-5. Minimum predict not-effect concentration for digested sludge (PNEC) and risk quotient (RQ) ratio calculated between the average concentration of PhACs in digested sludge and PNEC.

Groups	Pharmaceutical compounds	PNEC digested sludge, ng g^{-1} DM	References for minimum Kd and PNEC in water	RQ digested sludge
AIAPs	**Acetaminophen**	19	Verlicchi and Zambello, 2015; Tran et al., 2018	**2,69**
	Codeine	224	Verlicchi and Zambello, 2015; Verlicchi et al., 2012	0,07
	Diclofenac	0.36	Gonzalez-Gil et al., 2018; Orias and Perrodin, 2013	**169**
	Ibuprofen	0.1	Gonzalez-Gil et al., 2018; Tran et al., 2018	**15239**
	Indomethacin	97.5	Narumiya et al., 2013; Tran et al., 2018	0,46
	Ketoprofen	21.6	Verlicchi and Zambello, 2015; Orias and Perrodin, 2013	0,81
	Naproxen	3.63	Gonzalez-Gil et al., 2018; Tran et al., 2018	**13,88**
	Propyphenazone			
Antibiotics	**Clarithromycin**	1.58	Narumiya et al., 2013; Verlicchi et al., 2012	**30,4**
	Ofloxacin	25.3	Narumiya et al., 2013; Verlicchi et al., 2012	**876**
	Sulfadiazine			

	Sulfamethazine	75	Verlicchi and Zambello, 2015; Tran et al., 2018	**2,46**
	Sulfamethoxazole	0.162	Gonzalez-Gil et al., 2018; Tran et al., 2018	**56,2**
	Trimethoprim	0.394	Verlicchi and Zambello, 2015; Orias and Perrodin, 2013	**117**
Beta-blocker	**Atenolol**	220	Verlicchi and Zambello, 2015; Orias and Perrodin, 2013	0,07
	Metoprolol	1.8	Verlicchi and Zambello, 2015; Orias and Perrodin, 2013	**24,17**
	Propranolol	16.5	Verlicchi and Zambello, 2015;Orias and Perrodin, 2013	0,93
	Sotalol			
Diuretics	**Furosemide**	110	Verlicchi and Zambello, 2015; Orias and Perrodin, 2013	0,74
	Hydrochlorothiazide			
Lipid-regulators	**Bezafibrate**	4.6	Narumiya et al., 2013; Tran et al., 2018	**4,72**
	Fenofibrate			
	Gemfibrozil			
Psychiatric medications	**Carbamazepine**	40	Gonzalez-Gil et al., 2018; Orias and Perrodin, 2013	**5,28**
	Diazepam			
	Lorazepam	191	Verlicchi and Zambello, 2015; Orias and Perrodin, 2013	0,05
	Paroxetine	9680	Verlicchi and Zambello, 2015;	0,00

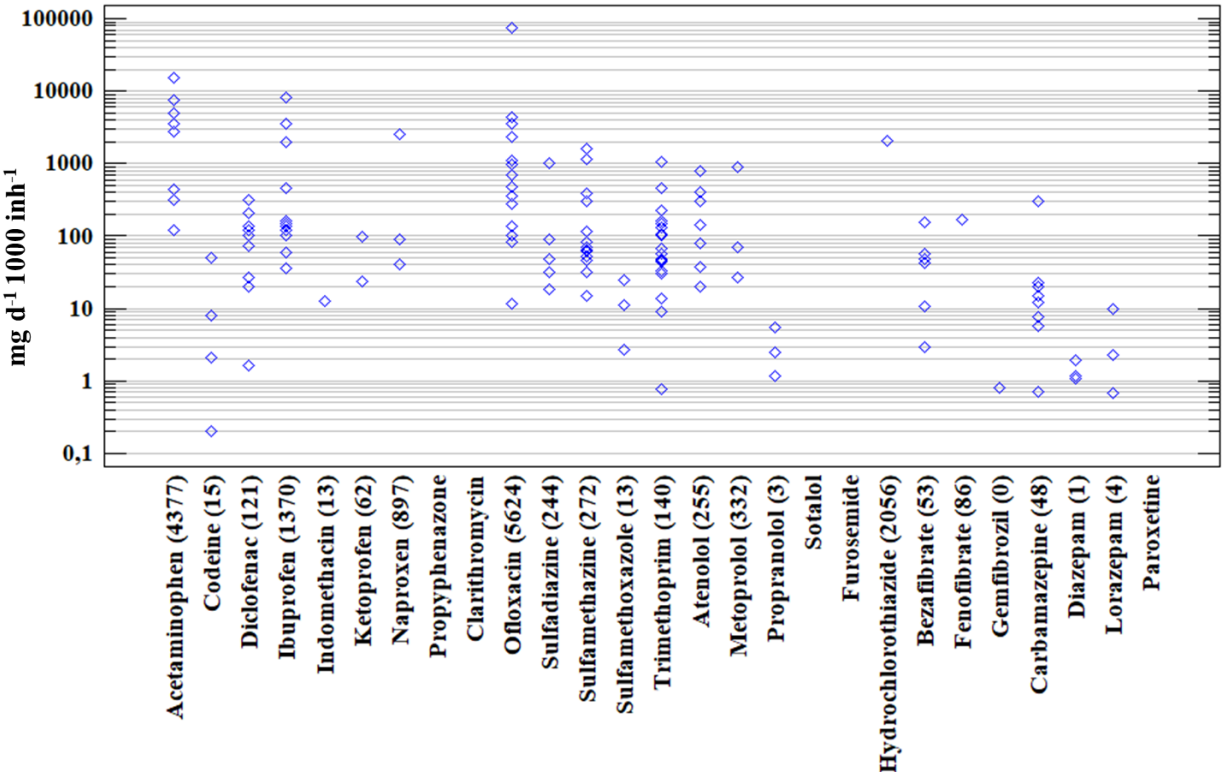
6.3. Daily mass load of the 27 selected PhACs in the wastewater influent and secondary effluent of municipal WWTPs

The daily influent mass load is a very important rate because it can be compared with the national consumption data and the predict concentration data of each compound, and give an idea about the consumption rate of each compound for the population than serve the WWTPs. Moreover, the average secondary effluent mass load of municipal WWTPs can give us an idea of the amount that can receive the environment every day.

Daily mass loads of the 27 selected PhACs for the influent and secondary effluent of municipal WWTPs revised in the literature are shown in Figure I-6 and Figure I-7 respectively. The corresponding average mass load values are in brackets after the name. The number of papers revised for each compound and their references are shown in Table I-S3. A total of 24 paper have been revised where the majority of the studies were done in Europe (15 papers) followed by Asia (6), North America (2) and Australia (1).

Referring to Figure I-6, the highest means influent mass loads (with data n≥3) were calculated for the antibiotic ofloxacin (5624 mg day^{-1} 1000inh^{-1}), and the AIAPs acetaminophen (4377 mg day^{-1} 1000inh^{-1}) and ibuprofen (1370 mg day^{-1} 1000inh^{-1}), with the highest absolute mass load values for ofloxacin (75075 mg day^{-1} 1000inh^{-1}), acetaminophen (15440 mg day^{-1} 1000inh^{-1}) and ibuprofen (8257 mg day^{-1} 1000inh^{-1}). The lowest average mass load (with data n≥3) were for the psychiatry medicaments diazepam (1 mg day^{-1} 1000inh^{-1}) and lorazepam (4 mg day^{-1} 1000inh^{-1}), and the beta-blocker propranolol (3 mg day^{-1} 1000inh^{-1}).

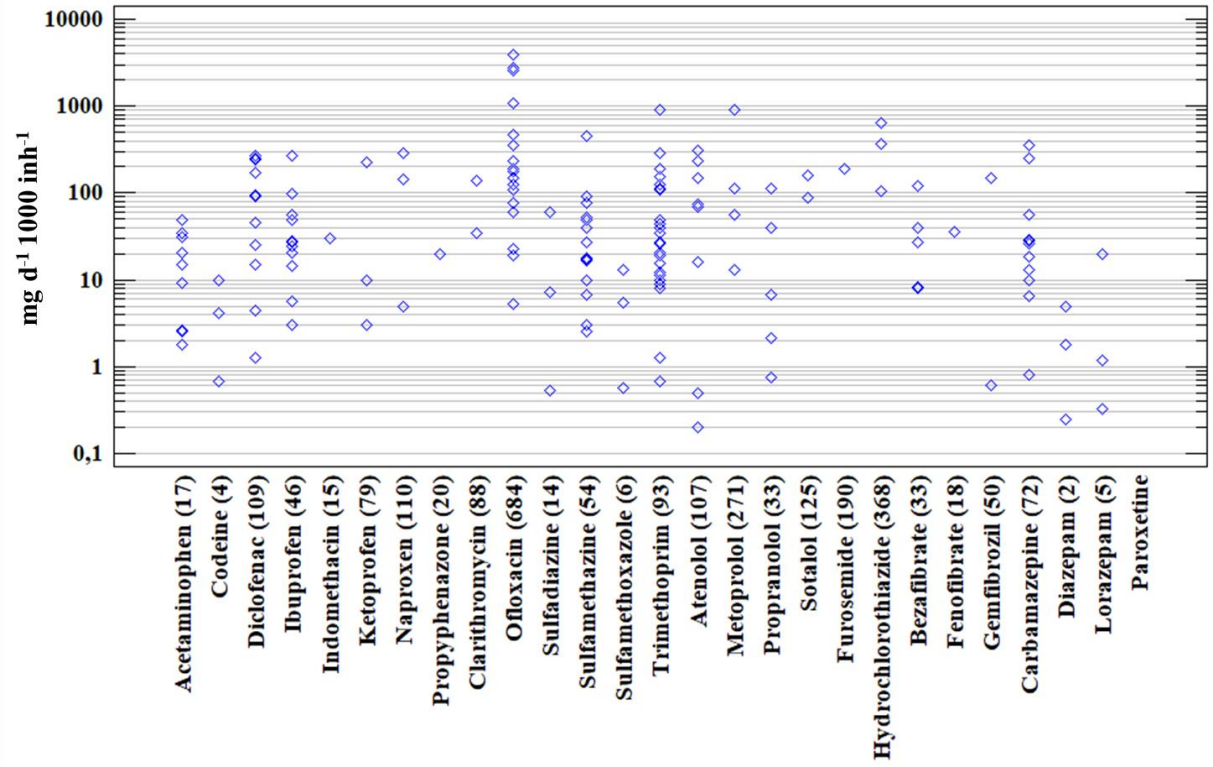
Figure I-6. Mass load (mg day^{-1} 1000 inh^{-1}) of the selected PhACs measured in the influent municipal wastewater of the WWTPs reported in the bibliography (Table I-S3). The corresponding average concentration values are in brackets after the name.



Referring to **Figure I-7,** the highest average effluent mass loads (with data n≥3) were calculated for the antibiotic ofloxacin (684 mg day^{-1} 1000inh^{-1}), the diuretic hydrochlorothiazide (368 mg day^{-1} 1000inh^{-1}) and the beta-clocker metoprolol (271 mg day^{-1} 1000inh^{-1}), with the highest absolute mass load values for ofloxacin (3953 mg day^{-1} 1000inh^{-1}), metoprolol (900 mg day^{-1} 1000inh^{-1}) and trimethoprim (898 mg day^{-1} 1000inh^{-1}). The lowest average mass load (with data n≥3) were found for the psychiatry medicament diazepam, lorazepam, the AIAPs codeine and the antibiotic

sulfamethoxazole. Considerably lower absolute and average values have been found for the majority mass load of PhACs in the secondary effluent compared to the mass load in the influent, highlighting the removal capacity of the WWTPs.

Figure I-7. Mass load (mg day^{-1} 1000 inh^{-1}) of the selected PhACs measured in the secondary effluent of the WWTPs reported in the bibliography (Table I-S3). The corresponding mean concentration values are in brackets after the name.



7. Biological treatments of municipal wastewater

7.1. Evolution of wastewater treatment technologies

In the 19th century, the industrial revolution concentrated the population in big cities with the consequent increase of industrial and urban wastewaters, which were discharged directly into rivers, producing many environmental problems. These contaminated waters created a serious risk for the humans and living organisms. Consequently, the science in wastewater treatment was born to solve these problems, apart from the first physico-chemical treatments (chemical precipitation, sedimentation, filtration, etc.), Alexander Mueller showed in 1865 for the first time that urban wastewaters could be purified by

living organisms in a filtration column (Pearse, 1938; Tiyasha et al., 2013). As a result, in the early 1880s the first biological wastewater treatment systems were based in a formation of a biofilm, such as trickling filters, intermittent filters and contact bed (Rodgers and Zhan, 2003; Rosso et al., 2011). Later in 1914, the conventional activated sludge system (CAS), which is the wastewater treatment process more used worldwide, was developed under the patent by Edward Arden and Willian T. Lockett. The authors observed that when the wastewater was storage in aerobic condition after a while, the liquid phase could be separated by sedimentation and replaced with new wastewater; in this way, the concentration of the solid phase could be increased, thus accelerating the process. This concentrated solid phase was identified as suspended activated biomass with microorganism and was the responsible for oxidation and consumption of dissolved organic matter (Tchobanoglous et al., 2003).

Figure I-8 shows the historical development of secondary treatment configuration of WWTPs based in activated sludge with the range data of the main operational parameters, such as sludge retention time (SRT), hydraulic retention time (HRT), mixed liquor suspended solid (MLSS) and returned activated sludge (RAS).

During the first half of 20^{th} century, the concept of biological oxygen demand (BOD_5) emerged to measure the concentration of organic matter, and the WWTPs were firstly designed to reduce this BOD_5 (configuration A, Figure I-8). From the second half of the 20th century, the rivers could not buffer the continuous discharge of nutrients (N and P) producing a considerable increase of the eutrophication process. To solve this problem, in the 1960s and 1970s, the engineers and microbiologists tried several modifications in the first CAS process to improve the nitrogen removal, they found that higher SRT and HRT (B configuration, Figure I-8) increase the nitrification process (ammonium was oxidized to nitrate). Therefore, the microbiologists found that the nitrification process was done by autotrophic bacteria, which need between two or three times more time to grow than heterotrophic bacteria (Henze et al., 2008; Tchobanoglous et al., 2003). At the same time, Ludzack and Ettinger demonstrated that the nitrate recycled from the secondary sedimentation tank (RAS) and combined with the secondary wastewater influent into an anoxic zone, can be reduce to nitrogen gas by several heterotrophic bacteria (Henze et al., 2008); in this sense, the modified Ludzack-Ettinger process was created for nitrogen removal with the possibility of phosphorous removal by chemical precipitation (C configuration, Figure I-8).

Additionally, the enhanced biological phosphorous removal was developed in the 1970s and patented in 1980s; it is a two-steps process in which heterotrophic polyphosphate accumulating organisms (PAOs) pass through an anaerobic environment followed by an aerobic one, wherein the aerobic zone the PAOs can take up and storage more phosphorous than they need. Consequently, the advance conventional activated sludge more used worldwide, the anaerobic/anoxic/aerobic (A^2O) process (configuration D, Figure I-8) was developed to remove biologically both nitrogen and phosphorous. Later, many biological nutrient removal configurations (BNR) were developed and used such as modified University of Capetown process (UCT), Five-Stage Bardenpho process or Johannesburg process.

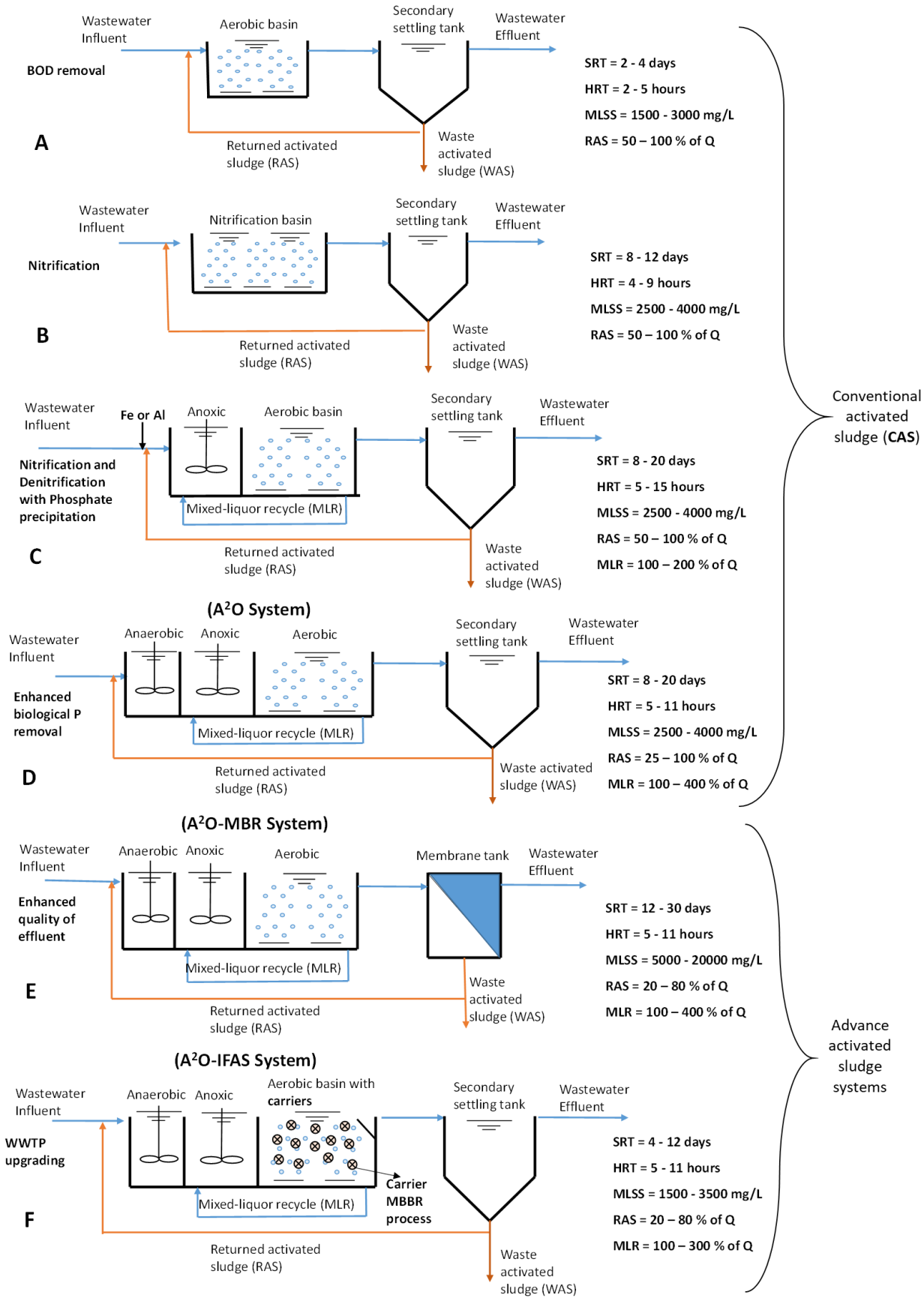
Due to the more stringer regulations on effluent quality, the need for water reuse, the increase in water price, the necessity of use less area implementation or upgrading the existing WWTPs, along with the better understanding and presence of emerging pollutant, new advanced wastewater treatments processes are needed to fulfil the legal requirement in WWTPs. Therefore, different technologies such as MBR process (E configuration, Figure I-8) and moving bed biofilm reactor (MBBR) process (C configuration) have been developed and are still being improved nowadays.

The first MBR installation with flat sheet ultrafiltration plate was done by Dorr-Oliver, Inc. in the 1970s. Afterward, during the 1970s and 1980s, it had considerable success in Japan but not in North America (Radjenović et al., 2008). The majority of installations were done for industrial wastewater before the 1990s, and after the development of submerged membranes in activated sludge, firstly introduced by Yamamoto et al. (1988), the number of MBRs municipal WWTPs experience accelerated growth worldwide, helped with the membrane price reduction.

Finally, in the late 1980s and early 1990s a new biofilm reactor technology "MBBR process" was developed in Norway (US Patent no. 5,458,779; European Patent no. 0,575,314) by the Norwegian authorities and the professor Ødegaard (di Biase et al., 2019; Ødegaard, 2006). It was first developed to address the needs of having small WWTPs that reduce the organic matter and nutrients efficiently and could be easy to operate and install in a small community (20-2000 people). The MBBR technology has several advantages over the other biofilm-based technologies and conventional activated sludge systems, such as the capacity of upgrading existing WWTPs with minimal additional costs, the need of lest sludge recirculation operating in IFAS mode, less clogging and no need to backwash compared to fixed-film reactors, more resistant to

variation in influent characteristics, etc. Accordingly, this technology has been used worldwide and become commercially successful, for instance, in 2014 more than 1200 WWTPs in at least 50 countries operated with MBBR technology (Biswas et al., 2014). Nowadays, the upgrade of CAS-WWTP with little additional cost can be done with the MBBR system in IFAS mode (F configuration, Figure I-8). This technology has the advantage of operating with suspended growth biomass (activated sludge) and attached growth biomass (biofilm microorganism) (di Biase et al., 2019).

Figure I-8. Historical development of the activated sludge process more used worldwide, from CAS systems (A, B, C and D) to MBR systems (E) and MBBR systems in IFAS mode (C). Typical operational variables are described.



7.2. Conventional activated sludge process – basic concepts

The systems try to amplify in a bioreactor the natural biodegradation and bioaccumulation process of organic matter and nutrients that occur in nature. Different microbial groups, which are responsible for the depuration process, assimilate and mineralize the dissolved organic matter and nutrients in a bioreactor continuously feed by new wastewater. These microorganisms (activated sludge) are separated by sedimentation in a secondary settling, where two flow are obtained; secondary effluent water and a secondary sludge. One part of sedimented secondary sludge is recirculated to the bioreactor to keep the microorganism concentration (MLSS) constant and the other goes to sludge stabilization process.

The main operational and design parameters are:

- **Hydraulic retention time (HRT, hours):** It is the average time that the wastewater is in contact with the activated sludge present in the bioreactor. It can be calculated by the influent flow (Q) and bioreactor volume (V).

  $$HRT = \frac{V}{Q}$$

- **Mixed liquor suspended solid (MLSS, mg day^{-1}):** It is the concentration of biomass in the biological reactor. It is composed by mixed liquor volatile suspended solids (MLVSS) which are usually considered as biomass and the mixed liquor fixed suspended solids ($MLSS_f$).
- **Food/microorganism ratio (F/M, Kg BOD_5 Kg $MLSS^{-1}$ day^{-1}):** It is the ratio between the influent organic load (kg BOD_5 d^{-1}) and the biomass concentration (MLSS or $MLSS_v$) in the bioreactor.

  $$F/M = \frac{BOD_{5\ influent}}{MLSS \times HRT}$$

- **Sludge retention time (SRT, days):** It is the average time that the activated sludge is in the bioreactor, it is also called sludge age. If the bioreactor works in a perfect mix and steady-state it can be calculated by means of wasted activated sludge flow (Q_w) and bioreactor volume.

  $$SRT = \frac{V}{Q_w}$$

- **Returned activated sludge ratio (RAS):** It is the return flow ratio over the influent flow (Q) of sedimented secondary sludge that is returned to the bioreactor to keep the required biomass concentration (MLSS) and F/M

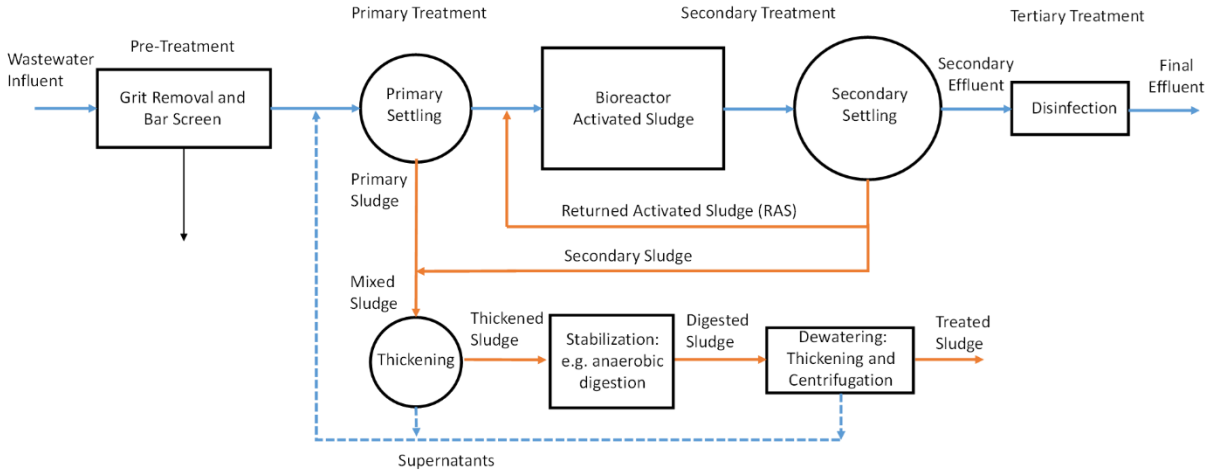
ratio to biodegrade the organic matter (BOD_5). It is calculated by a mass balance over the bioreactor.

- **Mixed liquor recycle ratio (MLR):** It is the return flow ratio from the effluent of the bioreactor to the influent of activated sludge that contain high concentration of nitrate for the removal of nitrogen. It is also called internal recycle. The value is linked to the nitrate effluent concentration.

Depending on the F/M ratio, there are three types of WWTP configuration with CAS process. First, activated sludge low loaded or oxidation ditch process, it is characterized for operate without primary sedimentation, the F/M ratio is between 0.05-0.15 $KgBOD_5\ KgMLVSS^{-1}\ day^{-1}$, high HRT (18-36 hours) and high SRT (20-30 days), it is mostly use for small WWTPs. Second, activated sludge medium loaded, this is the most used system worldwide in big WWTPs, which operate with F/M ratio between 0.2 – 0.6 $KgBOD_5\ KgMLVSS^{-1}\ day^{-1}$, medium HRT (5-11 hours) and medium SRT (5 – 15 days). Third, activated sludge high loaded, this configuration is used scarcely due to the low removal efficiency of BOD_5, which operated with F/M between 0.4 - 1.5 $KgBOD_5\ KgMLVSS^{-1}\ day^{-1}$, low HRT (2-4 hours) and low SRT (5-10 days).

WWTPs with activated sludge systems have been employed extensively worldwide for the treatment of urban and biodegradable industrial wastewater, mainly because they produce effluents that fit with the required quality standard at reasonable maintenance and operating cost. Figure I-9 shows a typical flow chart of a WWTP that operates with CAS.

Figure I-9. Typical flow diagram of municipal WWTPs with conventional activated sludge and anaerobic digestion of sewage sludge.



The raw influent wastewater firstly past through the pre-treatment process (bar screen, grit chamber, and oil and grease removal unit) to remove heavier and larger solids to protect the following process equipment. Subsequently, the settleable suspended solids are separated in the primary settling tank to reduce the organic load for the biological treatment. In the case of small WWTP, the use of primary sedimentation is omitted to reduce the management of primary sludge and amount of total sludge. The decanted wastewater goes to the activated sludge bioreactor where the microorganisms (activated sludge) adsorb, oxide, assimilate and mineralize the organic matter, nutrients and organic and inorganic pollutants, producing settleable flocs that are removed by sedimentation in the secondary settling. The final secondary effluent typically passes through a sand filter and disinfection process before discharge. Tertiary treatment can be possible if better water quality is need (e.g., reclaimed water, discharge in a sensible environment, etc.). The primary sludge and secondary sludge are thickened together or individually to increase de solid content (>3%). Later, the thickened sludge passes through a sludge stabilization process to remove pathogens and sludge volume after the dewatering process. Anaerobic digestion is the stabilization process mostly applied in big WWTP because it can produce biogas for renewable energy, while reduces the organic matter, destroys most pathogen present in sludge and reduces the sludge volume.

7.3. Moving bed biofilm reactor technology in municipal wastewater treatment-basic concepts

This technology is based in the formation of biomass in form of biofilm adhered inside of plastic carriers designed with a high surface area and similar water density that can be suspended and move freely around the whole bioreactor volume. The movement of carriers is done by air in the aerobic basin or by mechanical mixers in anaerobic or anoxic basin. This technology was originally invented as “pure” moving bed biofilm reactor (MBBR) without sludge recycling where the biomass manly develops on carriers, but an increased use and attention has been focused in hybrid MBBR or IFAS-systems in which carriers are added into the activated sludge bioreactors (Leyva-Díaz et al., 2017; Ødegaard et al., 2012).

The essence of MBBR process lies in the type of biofilm generated in the carriers, these are designed to provide an optimal surface area and dimension than give optimal cultivation of microorganisms when they are freely suspended in the water (McQuarrie

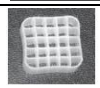
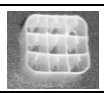
and Boltz, 2011). Besides, it should facilitate the requirements regarding the transfer of substrate and oxygen to microorganisms (Plattes et al., 2007).

Table I-6 summarizes the main characteristics and manufactures of some commercially available plastic carriers. The first carrier used in MBBR process were the K-series AnoxKaldnes^{TM}, they are the most used worldwide (di Biase et al., 2019). These carriers are made of polyethylene with a specific gravity between 0.94 and 0.96 g cm^{-3} which increase slightly when it is covered with biofilm. The effective surface area is the protected portion, normally the interior channels along with the media which have no contact with other carriers and where the biofilm primarily grows (McQuarrie and Boltz, 2011). Depending on the removal requirements, characteristics of the wastewater and process environment (e.g., aerobic, anoxic or anaerobic), the carrier used may change. For instance, for fast-growing aerobic heterotrophic microorganism biofilms, the carrier should have a wider opening to avoid clogging and effective surface area losses, such as in the K-series of AnoxKaldnes. Otherwise, for slow-growing autotrophic microorganism biofilm (e.g., in anammox and nitrification process) is preferable smaller opening and larger surface area such as in BiofilmChips or especially in recently developed Z series of AnoxKaldnes (di Biase et al., 2019).

In general terms, the MBBR process can be classified as pure MBBR system or hybrid MBBR system with the corresponding separation unit (e.g., membrane or settling tank). In pure MBBR systems the biomass mainly develops attached to the carriers and there is no recycling biomass from the separation unit. The biomass concentration correspond to the biofilm density (BD, mg L^{-1}) and the MLSS is similar to the total suspended solid (TSS) concentration of the influent (Leyva-Díaz et al., 2017). Compared with pure MBBR, the hybrid MBBR systems or IFAS-systems has both the attached biomass (BD) and the suspended biomass (MLSS), where there is a recycling biomass from the separation unit to the bioreactor to keep the working concentration biomass (MLSS) (Mannina and Viviani, 2009). The IFAS-system has gained more attention nowadays and this configuration have been shown more attractive and superior to pure MBBR systems (di Biase et al., 2019; Ødegaard et al., 2012).

During the last two decades and more recently, many studies have been done on IFAS-system to compare different carriers and to investigate the best operational parameter and performance, showing the efficiency of these systems for the removal of organic matter, nutrients and many organic micro-pollutants (Grandclément et al., 2017; Leyva-Díaz et al., 2020, 2017). This process includes the best characteristics of CAS and

Table I-6: Main characteristics of plastic carriers reported by the main manufactures.

Manufacturer	Name	Surface Area ($m^2 m^{-3}$)	Nominal Height;Diameter	Carrier Photograph
Veolia AnoxKaldnes™	K1	500	7.2 mm; 7.1 mm	
	K2	350	15 mm; 15 mm	
	K3	500	10 mm; 25 mm	
	K5	800	3.5 mm; 25 mm	
	C2	220	30 mm; 36 mm	
	F3	200	37 mm; 46 mm	
	BiofilmChip M	1200	2.2 mm; 48 mm	
	BiofilmChip P	900	3 mm; 45 mm	
	Z-200	-	n.d.; 30 mm	
	A-400	-	n.d.; 30 mm	
Headworks BIO (* Licensed by: Infilco Degremont, Inc.)	ActiveCell™ 450	402	15 mm; 22mm	
	ActiveCell™ 515	485	16 mm; 22mm	
	ActiveCell™ 920	485	15 x 15 x 10 mm	
AqWise	ABC4™	600	14 mm; 14 mm	
	ABC5™	650	12 mm; 12 mm	
Entex Technologies, Inc.	Bioportz™	589	14 mm; 18 mm	
Bio Proj Tecnologia Ambiental Ltda	Biobob®	-	60 mm; 45 mm	
Siemens Water Technologies Corp.	CM-10D™	750	9 mm; 13 mm	
Biowater Technology	BWT15™	828	15 x 15 x 5 mm	
	BWTX™	640	15 x 15 x 10 mm	

biofilter processes, lessening the settleability problems and fouling associated to pure MBBR and MBR systems, respectively (Leyva-Díaz et al., 2017; McQuarrie and Boltz, 2011). Indeed, this process became a very simple and efficient technology for upgrading overloaded WWTPs or design a new municipal WWTP (di Biase et al., 2019; Leyva-Díaz et al., 2017).

Table I-7 shows the main operative parameters reported in bibliography from the last two decades for full-scale and pilot-scale urban wastewater treatment plants mainly using IFAS-systems with a settling tank or membrane bioreactor as a separation system. The filling ratio is the volume fraction of the carrier over the total volume bioreactor. To ensure the proper mixing and reactor hydrodynamic it has been reported that the filling ratio must be below 70% (Ødegaard, 2006). The HRT correspond to the whole bioreactor, the SRT is done over the suspended biomass (MLSS) and the BD shows the attached biomass concentration in the bioreactor.

The filling ratio tested in the studies reported in Table I-7 for the pilot-scale IFAS ranged from between 0.29 to 0.66, being the most used below 0.5. Several authors such as Di Trapani et al. (2008), Mannina et al. (2007) and Martín-Pascual et al. (2012) reported that better performances have been observed for 0.35 filling ratio compare to 0.6, 0.5 and 0.66 filling ratio. Regarding the other operational variables, the HRT ranged from 4 to 63 hours, being the most used below 13 hours, and the SRT ranged from 2.8 to 65 days, being the most used below to 12 days. Concerning the suspended biomass concentration (MLSS) with settling tank in pilot-scale plants the values tasted ranged from 1718 to 5400 mg L^{-1}, being the most used values below 3500 mg L^{-1}, and with MBR as physical separation, the values range from 2042 to 6500 mg L^{-1}. Finally, concerning full-scale IFAS, the filling ratio reported ranged between 0.25 to 0.67, being the most used below 0.5, with SRT range from 5 to 17.5 days, being the most used below 10 days.

Table I-7. Operational parameters of hybrid MBBR system in IFAS process for full-scale and pilot-scale urban wast
pilot-scale pure MBBR and three full-scale pure MBBR which investigated PhACs removal.

System configuration	Physical Separation	Type of carrier	Filling ratio	HRT (hours)	SRT (days)	MLSS (mg L^{-1})	BD (mg
Full-scale IFAS	Settling tank	AnoxKaldnes K5	0.35	-	12.4	-	-
Full-scale IFAS	Settling tank	ActiveCell 450	0.35	-	9	-	-
Full-scale IFAS	Settling tank	ActiveCell 451	0.5	-	17.5	-	-
Full-scale IFAS	Settling tank	BiofilmChip P	0.38	-	6.7	-	-
Full-scale IFAS	Settling tank	AnoxKaldnes K1/K3	0.50-0.67	-	9.9	-	-
Full-scale IFAS	Settling tank	AnoxKaldnes K3	0.52	-	9.1	-	-
Full-scale IFAS	Settling tank	Polypropylene 25/15	0.25	9.5	5	5500	-
Full-scale IFAS	Settling tank	-		5	-	-	-
Pilot-scale IFAS	MBR	AnoxKaldnes K1	0.35	10	26	6500	-
Pilot-scale IFAS	MBR	Amitech®	0.15-0.40	-	65	-	-
Pilot-scale IFAS	MBR	AnoxKaldnes K1	0.35	6	21	2500	-
Pilot-scale Pure MBBR	-	AnoxKaldnes K5	0.5	6.73	-	-	3368
Semi-real-scale IFAS	MBR	Christian Stöhr®	0.5	13	20	5300	-
Semi-real-scale IFAS	MBR	Christian Stöhr®	0.5	13	10	2500	-
Pilot-scale IFAS	MBR	AnoxKaldnes K1	0.5	24	25	6000	2250
Pilot-scale IFAS	MBR	AnoxKaldnes K1	0.35	9.5	11.7	2458-2498	1250
Pilot-scale IFAS	MBR	AnoxKaldnes K1	0.35-0.5	10-24	8.56-56.53	2579-4594	4403
Pilot-scale IFAS	Settling tank	AnoxKaldnes K5	0.5	9	-	3120	2400
Pilot-scale IFAS	MBR	AnoxKaldnes K1	0.35	9.5	7.2	2042	998
Pilot-scale IFAS	Settling tank	Biobob	0.07-0.18	48-63	8.2	1560-2328	-

Full-scale IFAS	Settling tank	BiofilmChip M	0.30-0.40	12	-	7100	-
Full-scale Pure MBBR	Settling tank	AnoxKaldnes K1	0.50-0.60	6-7	-	-	1340
Full-scale Pure MBBR	Settling tank	AnoxKaldnes K1	0.40-0.60	11-12	-	-	5400
Full-scale Pure MBBR	Settling tank	AnoxKaldnes K1	30	35	-	-	1300
Pilot-scale IFAS	Settling tank	AnoxKaldnes K1	0.3	7.4	8.32	2000	3140
Pilot-scale IFAS	Settling tank	Bioportz	0.5	-	4_8	1740-2690	1031
Pilot-scale IFAS	Settling tank	AnoxKaldnes K1	0.29	4.04	2.8	2000	-
Pilot-scale IFAS	Settling tank	AnoxKaldnes K1	0.66	13	-	-	-
Pilot-scale IFAS	Settling tank	AnoxKaldnes K1	0.6	10	5.0-6.0	4800-5400	8240
Pilot-scale IFAS	Settling tank	AnoxKaldnes K1	0.37	5.8-6.0	4.2-8.2		-
Pilot-scale IFAS	Settling tank	AnoxKaldnes K1	0.35	-	-	3500-4900	-
Pilot-scale IFAS	Settling tank	AnoxKaldnes K1	0.66	-	-	3200-4300	-
Pilot-scale IFAS	Settling tank	AnoxKaldnes K1	-	-	4	1718	-

7.4. Performance comparison of IFAS-system with CAS and MBR technologies to treat urban wastewater

In order to find the best performance of organic matter and nutrients removal with the lower operational cost, numerous comparisons have been done between IFAS-system and CAS. Di Trapani et al. (2010) concluded that IFAS-systems showed better organic and ammonium removal than CAS with an improvement of the sludge settleability properties concerning CAS process. Araujo Junior et al. (2013) studied the addition of carriers in a CAS process and indicated that at 18% filling ratio the chemical oxygen demand (COD) and TSS removal were not affected, but the total nutrient (TN) removal was improved by 14% with a reduction of sludge waste production. Regarding settleability, Kim et al. (2010) indicated that an improvement of settleability and density in all suspended phases were due to a decrease in SRT from 8 days to 4 days, which was related to increased phosphorous content in biomass. Moreover, Di Trapani et al. (2013) stated that high ammonium removal is possible when a hybrid MBBR reactor is operated at low SRT with low temperatures, even though high SRT is needed in CAS for low temperature, it is because a large fraction of nitrification process will take place in the biofilm.

Generally, the CAS systems requires high volume reactors to achieve a good nitrification process, while the IFAS-system can improve the nitrification process and reduce the bioreactor volume, Güneş et al. (2019) compared a CAS wastewater plant with a pilot-scale IFAS plant to test the nitrogen removal efficiency. It was concluded that the footprint was reduced to 50%, which a reduction in the operational cost and capital investment. Moreover, considerably lower orthophosphate effluent level was obtained for IFAS plant than for CAS plant (0.7 mg L^{-1} vs 4.2 mg L^{-1}).

Similarly, Iannacone et al. (2019) showed that simultaneous nutrient removal is possible using low dissolved oxygen levels in MBBR process, resulting in lower reactors volume and mix liquor recycle reduction that decreases the operational and investment costs of the WWTP. Several authors and co-worker (Di Trapani et al., 2014; Leyva-Díaz et al., 2013; Mannina et al., 2018, 2017; Paetkau and Cicek, 2011) have found higher nitrification-denitrification levels in the hybrid system called IFAS-MBR compared to pure MBRs, conventional MBR and CAS, suggesting the occurrence of simultaneous nitrification-denitrification inside the biofilm (Mannina et al., 2017). Ashrafi et al. (2019) obtained in a hybrid 5-stage Bardenpho-MBBR process high removal efficiencies of

about 98.20%, 92.54%, 94.70% and 96.50% for total COD, TN, total phosphorus (TP) and ammonium, respectively, at comparatively short HRT (15.74 hours) and low nitrate recycle ratio (2). Finally, Bashar et al. (2018) concluded in a study about the cost-effectiveness of phosphorus removal processes that MBR is lest cost-effective with $60/lb.-P removed compared to IFAS at $42.22/lb.-P removed, this research considered maintenance costs, sludge disposal, chemicals and energy consumption.

All in all, the IFAS-systems improve CAS performance, reducing the operational cost and the overall footprint, therefore, it is an interesting option to upgrade CAS plant at relatively low cost. Comparing IFAS-systems with conventional MBR process, there is no clear performance differentiation, although IFAS-systems provide lower operational costs (Leyva-Díaz et al., 2020).

8. Anaerobic digestion of sewage sludge

8.1. Introduction

The disposal and treatment of sewage sludge in a municipal WWTPs poses a significant challenge because it represents about 50% of operating cost and 30-40% of the capital cost in many wastewater treatment facilities (Appels et al., 2008; Demirer and Othman, 2008). Dry matter (DM) weight per capita production of raw sewage sludge before processing is between 66 and 90 g per person per day, and after processing the output from WWTPs is theoretically evaluated between 45-56 g DM $cap.^{-1}day^{-1}$ (Ivanová et al., 2018; Tchobanoglous et al., 2003). The average value in 2017 for the EU28 was 58.9 g DM $cap.^{-1}$ d^{-1} with the highest values for Spain (160.7 g DM $cap.^{-1}$ d^{-1}), Austria (85.8 g DM $cap.^{-1}$ d^{-1}), and Portugal (87.6 g DM $cap.^{-1}$ d^{-1}). In fact, it represents approximately a yearly production of 21.5 kg DM per person, which production of 11 million tons of raw sewage sludge DM every year in the EU28 (Ivanová et al., 2018).

When sufficient raw sewage sludge (primary and secondary sludge) is produced especially for WWTPs with more than 20,000-30,000 p.ie., anaerobic digestion (AD) is the most widely process used for sludge stabilization, due to the production of biogas with high performance in the sludge stabilization, sludge reduction and pathogen reduction (Appels et al., 2008). The AD digesters are airtight tanks where the biogas formed has a high calorific value and it is considered as a renewable source. However, the conventional AD has some limitation such as partial decomposition of organic matter, slow reaction rate associated to high digester volume, vulnerability to various inhibitors, presence of

CO_2, H_2S and moisture in the biogas and the increase of heavy metals and organic micropollutants in the digested sludge (Appels et al., 2008). Several methods have been investigated over the conventional single-stage AD to increase the amount of organic matter digested and to enhance the overall process performance such as, sludge pre-treatments methods (i.e. thermal, alkaline and mechanical disruption, sonication or ozone) which are energy-intensive and costly (Demirer and Othman, 2008) or biological method such as two-stage AD where the digestion process occurred normally in two digesters in series.

8.2. Basic principles of anaerobic digestion process

The AD of organic matter present in the wastewater sludge is accomplished through biological conversion of suspended and soluble organic matter to methane and carbon dioxide in an oxygen-free environment. This process involves the coordinate and interdependent actions of several groups of microorganisms with different metabolism that consume and produce several substances through different and specific bioreactions Figure I-10). Depending of the microorganism type and substrate biodegraded, the AD process is divided in four different steps: hydrolysis, fermentation or acidogenesis, acetogenesis and methanogenesis as shown in Figure I-10.

The hydrolysis is the first step needed to degrade both insoluble organic matter and high molecular weight compounds into a soluble organic form that can be utilized by the acidogenic bacteria (e.g., amino sugars, fatty acids, alcohols, etc.). This process is carried out by extracellular enzymes produced and excreted by the hydrolytic/acidogenic bacteria population for this specific purpose (Parkin and Owen, 1986; Pavlostathis and Giraldo-Gomez, 1991). The overall digestion is not possible unless the hydrolysis step is operating properly. Therefore, the overall rate is generally limited by the hydrolysis rate of complex organic substrates such as waste activated sludge (Appels et al., 2008; Parkin and Owen, 1986). The grade and rate of hydrolysis process depend on the size and type of particulate organic matter, the concentration of hydrolytic biomass and different environmental factors. Besides, not all organic matter (around 30 to 40% of total solids) can be hydrolysed to simple compounds due to complex nonhydrolyzable linkages, inaccessibility and structure of the biomass substrate (Parkin and Owen, 1986; Tchobanoglous et al., 2003).

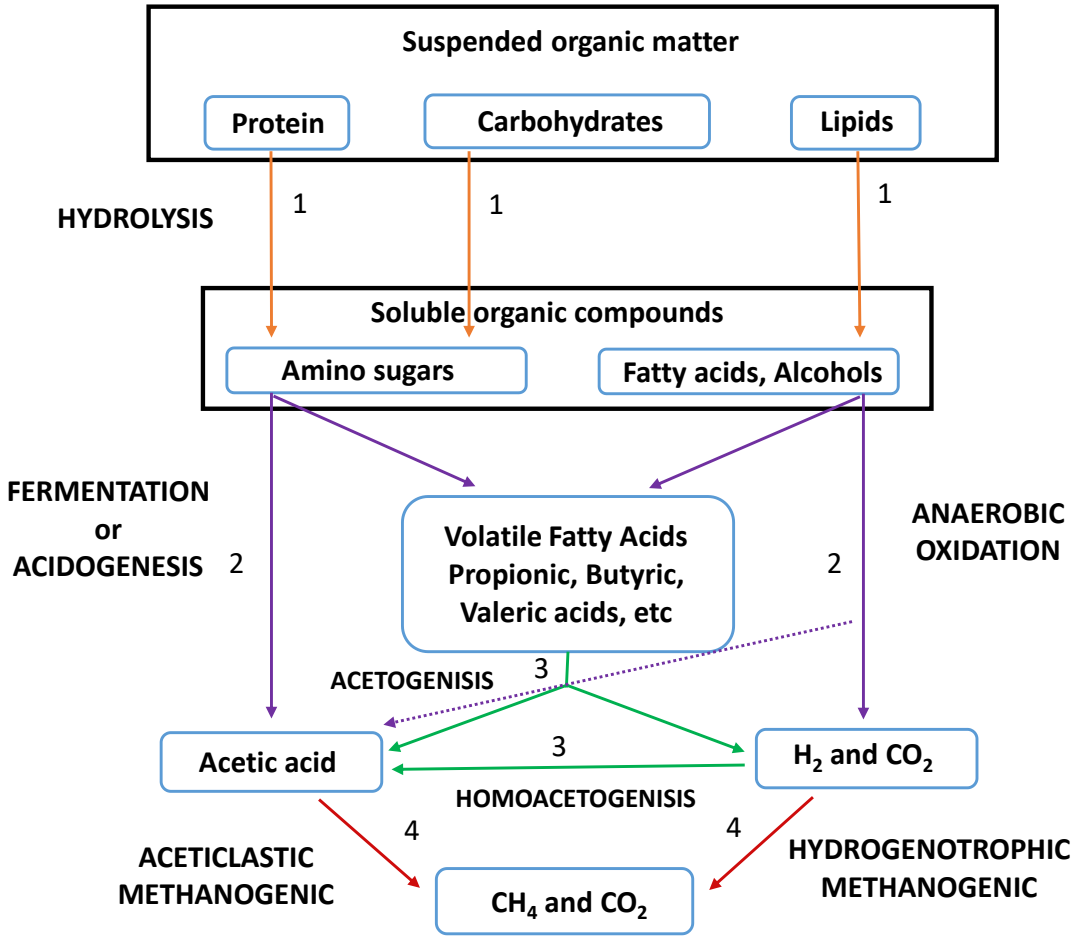
Once the complex organics are hydrolysed, the soluble organic molecules are fermented by several fermentative microorganisms producing lower molecular weight

compounds such as volatile fatty acids, acetic acid, H_2 and carbon dioxide. This step is commonly called the acidogenic or fermentation phase and is carried out by acidogenic bacteria population that may be facultative anaerobes, strict or obligate anaerobes and combination of both (Parkin and Owen, 1986). The activity of some acidogenic bacteria depends on H_2 concentration, where high concentration ($>10^{-4}$ atm of partial pressure) may cause inhibition. Hence, the continuous removal of H_2 by the hydrogenotrophic methanogenic archaea stimulated the action of the acidogenic bacteria. No many inhibitors have been described for this step, highlighting only the long-chain fatty acids (Angelidaki et al., 1999).

A special group of bacteria called syntrophic acetogenic bacteria are responsible for the production of acetic acid from the last products of the acidogenic phase. The production is carried out through two different routes and substrates, from volatile fatty acids to produce acetic acid and H_2 by acetogenic bacteria and production from carbon dioxide and H_2 to synthesize acetic acid by homoacetogenic bacteria (Figure I-10). The main point of these microorganisms in the digestion process is to produce acetic acid, carbon dioxide and H_2 for the methanogenic archaea. Similar to some acidogenic bacteria, the acetogenic bacteria are sensible to high level of H_2 concentration, being necessary that the H_2 consumption rate was equal or superior to the H_2 generation rate to prevent H_2 accumulation and digestion failure.

The sludge stabilization finally occurs during the methanogenic phase by the methanogenic archaea, which are responsible for the methane formation from the substrate with one or two carbon atoms produced in the previous phases. There are two main groups of methanogenic archaea (Figure I-10); hydrogenotrophic methanogenic archaea which consume H_2, CO_2 and acid formic; and aceticlastic methanogenic archaea which consume acetic acid, methanol and some methylamines. The presence of hydrogenotrophic archaea is essential to keep the correct level of H_2 concentration for the acidogenic and acetogenic bacteria. For AD of sewage sludge, approximately 72% of methane formed comes from acetate cleavage (aceticlastic archaea) and 28% results from reduction of CO_2 using hydrogen as the energy source (Ali Shah et al., 2014; Parkin and Owen, 1986). Several compounds have been described as inhibitors for the growth of methanogenic microorganisms such as volatile fatty acids, ammonium and oxygen molecular or even nitrates because they are strict anaerobes.

Figure I-10. Subsequent phases of the anaerobic digestion process. 1) Hydrolytic bacteria; 2) Acidogenic bacteria; 3) Acetogenic bacteria; 4) Methanogenic archaea. Adapted from Pavlostathis and Giraldo-Gomez (1991).



8.3. Factors affecting the rates of different steps of the anaerobic digestion process

The most important parameters that affect the rate and performance of the different steps of the AD process are described in the following points.

8.3.1. Solid and hydraulic retention time

The hydraulic retention time (HRT) is the average time that the liquid is kept in the digester and solid retention time (SRT) is the average time that the solids or biomass stay in the digester. SRT is operationally defined as the mass contained in the digester divided by the mass of solid wasted from the system per day. Commonly, the HRT is equal to SRT in sewage sludge high-rate digesters because there is no separation between the solid and liquid phase (SRT=HRT=volume/flow=V/Q).

The SRT is one of the key factors for microorganism's growth, where the four subsequent steps of the AD process are directly related to the SRT. To ensure efficient AD process, a sufficient SRT is needed to prevent washout of bacteria and archaea population. Therefore, cell growth must be equal or superior to cell removal to avoid process failure and ensure steady-state (Appels et al., 2008; Parkin and Owen, 1986).

SRT is recognized as the most important operating parameter for systems design and performance of AD process because SRT defines more accurately the relationship between operating performance (gas production, volatile solid removal, organic loading rate) and microorganisms' population (Appels et al., 2008; Parkin and Owen, 1986; Pavlostathis and Giraldo-Gomez, 1991).

The typical SRT for mesophilic AD process (35ºC) should be higher than 12 days and it is recommended not be less than 10 days for satisfactory methane production (Appels et al., 2008; Xu et al., 2020). Although the volatile solid removal increases with the increase of SRT, it does not always result in high biogas production. For instance, Nges and Liu (2010) found that the methane production rate could be improved by shortening from 35 days to 12 days for the AD process of WAS without comprising the volatile solid removal. Therefore, an optimal SRT should be found to decrease the operational and design cost.

8.3.2. Temperature

The temperature is a fundamental parameter in the AD process because small changes of it can influence the growth rate and metabolism of microorganisms, with the consequent change of the population dynamics in the digester. Therefore, the temperature has an important effect in the gas solubility, in salts solubility and in the equilibrium of different chemical reactions. Anaerobic digestion failure can occur at temperature changes in excess of 1ºC day^{-1} for high-rate digester, so it is important to maintain a stable operating temperature (Appels et al., 2008).

The AD process can work at different temperature ranges: psychrophilic (5-20 ºC), mesophilic (30-40 ºC) and thermophilic (50-60 ºC), where the optimum temperature ranges commonly used for AD of sewage sludge correspond between 35-37 ºC (mesophilic) and 55-57 ºC (thermophilic). In general, higher temperature (thermophilic range) increase the bioreaction rate and volatile solid removal with the consequent increase of biogas production (Bolzonella et al., 2012, 2003; Moen et al., 2003), as well as, increases the sludge stabilization and pathogen removal compared to mesophilic

(Watanabe et al., 1997). However, operating at high temperature (thermophilic) increases the fraction of free ammonia, the pK_a and volatile free acid (VFA), which can inhibit some micro-organisms and reduce the capacity to control the process (Appels et al., 2008).

8.3.3. Alkalinity, pH and VFA/alkalinity ratio

The pH value gives the concentration of protons in dissolution. Although the pH can be measured simply and continuously, this is no unique control parameter because it is influenced by multiple factors. For instance, the pH can decrease with the accumulation of VFA but it can be higher or lower depending upon the alkalinity. Similarly, the pH can be controlled by the CO_2 concentration in the gas phase and the HCO_3-alkalinity of the liquid phase. However, the VFA/alkalinity ratio is a more important control parameter than the pH, VFA and alkalinity level by itself. A VFA/alkalinity ratio below 0.7 should be maintained for stable digestion process (Appels et al., 2008).

Each group of microorganisms has an optimum pH range operation. The hydrolytic bacteria works properly between 7.2-7.4, the acidogenic between 5.5-6.5, the acetogenic between 7-7.2 and the methanogenic between 6.5-7.5 (Appels et al., 2008; Yu and Fang, 2002). The methanogenic archaea are extremely sensitive to abrupt changes of pH that normally occur when there is a big increase of the organic loading rate, producing an accumulation of VFA in the digester because the acidogenic and acetogenic bacteria have higher growth rate than the methanogenic archaea. However, there are some species like *Methanosarcina sp*, with have higher growth rate (i.e. in order of 1.0 to 1.2 days doubling times) and are more resistant to quickly pH changes (between 0.8-1 units from optimum value) compared to the other methanogens, which tend to be inhibit by a pH shock of 0.5 units and have doubling times of minimum 4-6 days (Ali Shah et al., 2014; Maspolim et al., 2015a; Yang et al., 2014).

8.3.4. Organic loading rate

The organic loading rate (OLR, Kg VS $m^{-3}d^{-1}$) is an important operational and design parameter related to the SRT and influent volatile solid concentration that affect microbiota diversity and efficiency of the AD process (Saha et al., 2020). The digesters should be fed uniformly and continuously to help maintain steady-state conditions and reduce shock loading, especially for the most sensitive methanogenic archaea.

To some extent, the AD process efficiency can be enhanced with the increase of OLR. Lu et al. (2015) found that a digester with the same SRT (20 days) but higher OLR (5.6 vs 1.4 Kg VS $m^{-3}d^{-1}$) and high solid content (24 vs 6 %) achieved higher volatile solid removal and biogas production, which are related to the increase of several key bacteria and methanogenic archaea that enhance the hydrolysis, acidification and methanogenesis of sewage sludge. Additionally, Nges and Liu (2010) found that the methane production rate could be enhanced when the OLR increase by shortening the SRT. However, high OLR is associated to the decrease of pH due to the increase of VFA that can inhibit the activity of the methanogenic archaea (Appels et al., 2008; Saha et al., 2020; Xu et al., 2020).

For high-rate AD process of sewage sludge, the OLR should be between 1.6-4.8 Kg VS $m^{-3}d^{-1}$ with a sludge concentration between 4-7%. Lower solids loading decrease the efficiency of the process and higher OLR and sludge concentration (>7%) could increase the possibility of process failure (Appels et al., 2008; Xu et al., 2020).

8.3.5. Feed characteristic of sewage sludge

WWTPs produce two types of sewage sludge, primary sludge (PS) coming from the primary sedimentation process and waste activated sludge (WAS) coming from the secondary sedimentation. Commonly, it is firstly thickened and secondly mixed to be digested in the AD process. In some WWTPs the amount of carbon in the influent is not enough to perform properly the biological nutrient removal (BNR), as a consequence, the primary sedimentation tank is generally absent to preserve all the organic matter for the denitrification process. Moreover, long SRT is applied (>15 days) in the activated sludge which produces only low biodegradable WAS for the AD process (Bolzonella et al., 2005; Gonzalez et al., 2018; Xu et al., 2020).

Table I- shows the operational parameters and performance of the different AD process configurations to treat WAS, PS and a mix of both. The typical values reported in the literature for volatile solid removal (VSR) of PS is between 40-70% (Parkin and Owen, 1986). For instance, Yang et al. (2016) reported 69, 74 and 76 % of VSR for PS in a pilot-scale plant operating in a single-stage mesophilic AD process at 15, 20 and 30 days of SRT respectively. However, the anaerobically biodegradation of WAS is generally reported less than half of PS and other organic waste (Parkin and Owen, 1986; Xu et al., 2020). The typical values reported for VSR of WAS is often less than 35% in single-stage mesophilic digesters at 20-30 days of SRT (Gonzalez et al., 2018; Parkin and

Owen, 1986; Xu et al., 2020), For instance, Bolzonella et al. (2005) reported a VSR between 15-27 % (Table I-) of five full-scale single-stage digesters with SRT between 20-40 days, treating WAS coming from biological nutrient removal WWTPs. Therefore, the specific methane production was between 0.31-0.37 m^3CH_4 Kg VS destroyed^{-1} when the typical values for mixed sludge are in the range of 0.45-0.70 m^3CH_4 Kg VS destroyed^{-1}. The authors found a clear relationship between the specific methane production and the SRT applied in the activated-sludge process of the WWTPs: the higher the SRT the lower the specific methane production.

To overcome the poor digestion performance of WAS, an increase of research has been done during the last decade with the aim of increasing the digestion rate (Xu et al., 2020). For instance, better VSR values for WAS were obtained operating at thermophilic AD process (Table I-) (Bolzonella et al., 2012) or two-stage AD process (Bolzonella et al., 2012; Ghosh et al., 1995; L.-J. Wu et al., 2016).

To conclude, WAS is intrinsically less anaerobic biodegradable than PS, where the AD process of both mixed can improve the poor performance of WAS. For instance, the VSR for mixed sludge (PS+WAS) reported in Table I- is between 33-69 % for mesophilic AD process with a specific methane production rate between 0.3-0.72 m^3CH_4 Kg VS destroyed^{-1}.

8.4. Conventional high-rate single-stage AD vs two-stage AD

The high-rate single-stage AD is the configuration most used nowadays in WWTPs for the treatment of sewage sludge. In a high-rate digester, the influent raw sludge should be fed continuously and uniformly, and the sludge inside the digester must be completely mixed and heated. A typical high-rate digester should have an OLR in the range of 1.6-4.8 KgVS $m^{-3}d^{-1}$, an SRT between 10-20 days, and a mixed total solid (TS) concentration between 4-7 % that can give a specific methane production (SMP) between 0.45-0.70 m^3 Kg CH_4 VS destroyed^{-1}, or 0.30-0.50 Kg CH_4 VS added^{-1} (Appels et al., 2008).

Most high-rate digesters are operated in the mesophilic range (35-37ºC). A digester working in thermophilic range (50-57ºC) can increase solid reductions, increases the removal of pathogenic organisms, and improves the dewatering process. However, those advantages are not usually good enough to overcome the main disadvantages, such as, higher energy requirement, lower quality supernatant, higher odour, a higher fraction of free ammonia and VFA, and lower capacity to control the process (Appels et al., 2008; Ghosh et al., 1995).

Due to the poor anaerobic biodegradability of WAS reported in the literature that requires large digestion tanks and the input of large heating energy with other additional drawbacks, such as, foaming problems, low start-ups and unstable fermentation, the two-stage AD has been proposed to overcome the shortcomings of conventional single-stage AD (Ghosh, 1987; Ghosh et al., 1995; Leite et al., 2016; Oles et al., 1997; Xu et al., 2020).

The two-stage AD process was developed due to the different growth rate and optimal pH for the acidogenic and methanogenic organisms that allow the selection and enrichment of different microbiota in each digester (Bhattacharya et al., 1996; Demirel and Yenigün, 2002; Ghosh, 1987; Ghosh and Donald L. Klass, 1982; Sidhu et al., 2013; Smith et al., 2017). The two-stage AD has some advantage over the conventional high-rate single-stage AD processes, such as preventing organic load and pH shock to the most sensible methanogenic organism, the increase stability of the process and the increase of volatile solid removal. Thus, several authors suggested that the AD digester could be more cost-efficient and smaller than one-stage conventional AD (Bhattacharya et al., 1996; Bolzonella et al., 2003, 2012; Demirel and Yenigün, 2002; Demirer and Othman, 2008; Ghosh, 1987; Ghosh et al., 1995; Leite et al., 2016; Maspolim et al., 2015a; Oles et al., 1997; Smith et al., 2017; Watts et al., 2006).

To find the main operational parameter and different performance between conventional AD and two-stage AD, a literature review is presented in Table I-8. It shows the main operational parameters and performance reported in the literature for full, pilot and lab-scale digesters treating WAS, PS or a mix of both in different AD configuration, such as mesophilic and thermophilic single-stage (MAD and TAD, respectively) and two-stage AD with different temperature operation in each digester (meso/thermo, thermo/meso, meso/meso or thermo/thermo). Table I-8 reports data from 26 full-scale digesters (10 single-stage MAD, 2 single-stage TAD and 14 two-stage AD), and 42 pilots/lab-scale digester (18 single-stage MAD, 10 single-stage TAD and 14 two-stage AD). It shows data from 18 different papers where 10 papers compare the performance between conventional single-stage and two-stage AD. It is important to compere the biogas production rate (BPR), the specific methane production (SMP) and VSR rate performance of different AD processes configuration with digesters that operate at the same operating parameter, such as SRT, temperature, TS, percentage of volatile solids (TS/VS), OLR and type of raw sludge (PS, WAS or mixed) because the change of each factor can change the rate of the AD process.

For instance, Ghosh (1987) investigated pairs of two-stage and single-stage AD in pilot-scale digesters at 35ºC and 55ºC with SRT's of 15, 7 and 3 days. He stated that the two-stage AD process is better than single-stage under all test conditions based on BPR, SMP, VSR and unconverted volatile acids in the effluent. The meso-meso two-stage AD run exhibited the best performance in all respects and more specific the meso-meso two-stage AD run with 2 days of SRT in the acidogenic digester (AcD) and 13 days in the methanogenic digester (MD) which produced a global BPR of 0.78 L L^{-1} d^{-1} and a SMP of 1.10 m^3CH_4 KgVSdestroyed^{-1} (Table I-8). Moreover, Ghosh et al. (1995) demonstrated firstly in a pilot-scale digester and secondly in a full-scale digester that the mesophilic two-stage AD process of WAS produced by DuPage County WWTP was considerably better than the old single-stage AD of DuPage (the existing single-stage digester was converted to a two-stage AD process). The pilot-scale mesophilic two-stage AD obtained a total VSR of 71 % and BPR of 2.2 L L^{-1} d^{-1} in only 12 days of SRT (3 days AcD and 9 days MD) and 4.7 KgVS $m^{-3}d^{-1}$ of organic loading rate without any evidence of digester foaming that was the main problem of the old single-stage AD of DuPage. They examined also the thermophilic acid-phase digestion that was slightly better but it was ruled out because of odour problems and the input of additional heating. The final operational parameter and performance of the new full-scale mesophilic two-stage AD are shown in Table I-8. Results of this research showed that good separation of hydrolytic-acidification and acetogenic-methanogenic phases can be achieved with not digester foaming, higher BPR (1.89 L L^{-1} d^{-1}) and even lower H_2S production.

Oles et al. (1997) reported the operational parameter and performance of six full-scale thermophilic/mesophilic two-stage AD treating mixed sewage sludge in Germany (Table I-8). These full-scale digesters cover a broad range of treatment plant sizes between 14,000 and 1.6 million p.ie. A comparison was possible between single-stage and two-stage AD in the WWTP at Cologne, with an SRT between 25-27 days the VSR rate could be considerably increase from 48% to 60% under thermophilic/mesophilic two-stage AD conditions. This improved the specific gas production by 16.5% and gave considerable saving in final sludge disposal and dewatering. The authors recommended 2-3 days SRT in the thermophilic stage (AcD) at 55-60ºC and 12-15 days SRT in the mesophilic stage (MD) at 35-37ºC, the TS should not exceed 6% due to the exponential increase of sludge viscosity.

Cheunbarn and Pagilla (2000) obtained in a lab-scale digester higher VSR rate (61%) in thermophilic/mesophilic two stage AD process compared with the control

single-stage MAD (50%) but the SMP were similar in both systems. Moreover, Watts et al. (2006) operated a thermophilic/mesophilic two-stage AD pilot-plant with solely WAS from a BNR plant. The AcD (SRT 2 days) was operated at 47, 54 and 60 ºC and the MD (SRT 15 days) at 37ºC. A control single-stage MAD (37ºC) was run with SRT 17 days for comparison. The digesters were operated at low total OLR (0.9 KgVS $m^{-3}d^{-1}$). Only the two-stage AD system with higher thermophilic temperature (60 ºC) of AcD achieved significant higher VSR (35 vs 24 %) and BPR (0.30 vs 0.25 $LL^{-1}d^{-1}$) compared with the control single-stage MAD process. The authors concluded that a truly thermophilic (≥ 60 ºC) stage is essential to achieve high VSR rates for treat poor anaerobic biodegradable WAS. The MD (second-stage) efficiently removed the excess of VFAs produced by the thermophilic AcD to low levels and increased the BPR compared to the control single-stage digester (Table I-8). Furthermore, Bolzonella et al. (2012) also found in a two-year pilot-scale experimental trial that the digestion of WAS was improved when the temperature operation was increased at high rate (OLR of 2.2-2.3 KgVS $m^{-3}d^{-1}$ and SRT of 20 days). The VSR rate increased from 36% in single-stage MAD (35 ºC) to 48% in single-stage TAD (55 ºC), and 55% in thermophilic two-stage AD (65+55 ºC). As a consequence, the BPR and the SMP also were increased with the temperature (Table I-8).

Maspolim et al. (2015a) compared the reactor performance and respective microbial community dynamics in the single-stage MAD and mesophilic two-stage AD systems, treating mixed sewage sludge at three different global SRT (30, 20 and 12 days). The authors concluded that the VSR rate and methane production of two-stage AD was maintained when was operated at 30, 20 and 12 days, while the single-stage MAD process performance deteriorated when the SRT was reduced from 20 to 10 days.

L.-J. Wu et al. (2016) studied in a lab-scale plant the different performance and microbiota diversity of hyper-thermophilic (70 ºC)-mesophilic (30 ºC) two-stage process (6 days of AcD and 24 days MD of SRT) and single-stage MAD process of WAS at 30 days of SRT. The VSR rate improved only over 10% in the two-stage AD but there was no significant difference in BPR between them. However, Leite et al. (2016) reported a clear increase in terms of VSR (34% vs 38%), BPR (0.50 vs 0.55 $LL^{-1}d^{-1}$) and SMP (0.56 vs 0.67 CH_4 KgVSdestroyed^{-1}) in a pilot-scale thermophilic two-stage AD compared to the control single-stage TAD, treating WAS at a high rate (6.2% of TS and 2.2 KgVS $m^{-3}d^{-1}$). The author estimated 15% more energy production and the installation of an additional AcD or hydrolytic reactor can be paid back in some 3 years.

Finally, Smith et al. (2017) reported the operational parameters, performance and microbial community structure of six full-scale two-stage AD facilities and four conventional single-stage AD facilities (Table I-8). Notwithstanding that a real comparison between different AD process configurations should be done with a similar substrate and operational condition, VSR and SMP were similar in both systems configuration, though the thermophilic second-stage digesters reported have the lowest VSR rate. The authors concluded that nearly complete phase separation was achieved in two-stage AD with few syntrophic bacteria or methanogens detected in the AcD, and the two-stage AD process exhibited greater microbial diversity than conventional single-stage AD, likely due to more diverse populations of hydrolytic and acidogenic bacteria in the AcD. They recommend running the methanogenic digester (second-stage) at mesophilic temperature because higher relative abundances of syntrophic bacteria and methanogens were found in mesophilic digesters, and lower energy requirements are needed for digester heating.

Table I-8. Operational parameters and performance of single-stage mesophilic anaerobic digestion (MAD), thermoph
AD process for waste activated sludge (WAS), primary sludge (PS) or mixed of both. BPR: biogas production rate; OL
VS/TS: volatile solid percent from TS; SMP: specific methane production; VSR: volatile solid removal. 1 plus fat, oil a

Type of AD process		Operational parameters						Perform	
System configuration	**Scale**	**Sludge feed type**	**SRT, days**	**T ºC**	**TS %**	**VS/TS %**	**OLR, Kg VS $m^{-3}d^{-1}$**	**VSR %**	**BPR L L^{-1} d^{-1}**
Single-stage MAD	Full-scale	Mixed	11	35	6.5	82	2.9	69	-
Single-stage MAD	Full-scale	Mixed[1]	11	35	6.5	82	2.9	68	-
Single-stage MAD	Full-scale	Mixed	27	35	4.7	77	1.5	41	-
Single-stage MAD	Full-scale	WAS	-	35	4.6	78	NA	59	-
Two-stage AD	Full-scale	Mixed	4/33	35/35	6.4	81	12.1/2.1	22 / - / 61	-
Two-stage AD	Full-scale	WAS	1.5/-	35/55	5.6	77	20.7/-	11 /41	-
Two-stage AD	Full-scale	Mixed	2.3/27	35/35	4.9	82	17.8/1.5	25 / -	-
Two-stage AD	Full-scale	Mixed	2.3/-	35/55	4.9	82	17.8/-	25 / - / 56	-
Two-stage AD	Full-scale	Mixed	2 / 35	35/35	4.5	82	28.0/1.0	23 / - / 62	-
Two-stage AD	Full-scale	Mixed	2.5/24	35/35	4.2	86	12.2/1.3	26 / - / 58	-
Two-stage AD	Full-scale	Mixed	1.6/26	35/35	4.2	86	12.5/1.0	19 / - / 68	-
Single-stage MAD	Pilot-scale	Mixed	20	35	-	-	1.1	45	-
Single-stage MAD	Pilot-scale	Mixed	15	35	-	-	1.4	33	-
Single-stage TAD	Pilot-scale	Mixed	20	54	-	-	1.1	49	-
Single-stage TAD	Pilot-scale	Mixed	15	54	-	-	1.4	39	-
Single-stage MAD	Lab-scale	Mixed	20	37	2.8	78	2.3	62	1.05
Single-stage MAD	Lab-scale	Mixed	30	37	2.8	78	1.3	58	0.55

System configuration	Scale	Sludge feed type	SRT, days	T ºC	TS %	VS/TS %	OLR, Kg VS $m^{-3}d^{-1}$	VSR %	BPR L L^{-1} d^{-1}
Single-stage TAD	Lab-scale	Mixed	20	55	2.8	78	2.3	54	0.91
Single-stage TAD	Lab-scale	Mixed	30	55	2.8	78	1.3	50	0.41
Single-stage TAD	Pilot-scale	WAS	20	55	6.2	68	2.2	34	0.50
Two-stage AD	Pilot-scale	WAS	2/18	55/55	6.2	68	14.5/2.2	22/38	0.87/0.55
Single-stage MAD	Lab-scale	WAS	30	35	4.7	74	1.2	38	0.45
Two-stage AD	Lab-scale	WAS	6/24	70/35	4.7	74	5.77/1.04	27/36/52	0.40/0.42/
Single-stage MAD	Pilot-scale	PS	15	35	2.5	89	1.5	69	-
Single-stage MAD	Pilot-scale	PS	20	35	2.5	89	1.1	74	-
Single-stage MAD	Pilot-scale	PS	30	35	2.5	89	0.7	76	-
Single-stage MAD	Pilot-scale	Mixed	30	35	3.2	80	0.9	32	0.63
Single-stage MAD	Pilot-scale	Mixed	20	35	3.2	80	1.3	32	0.64
Single-stage MAD	Pilot-scale	Mixed	12	35	3.2	80	2.1	26	0.53
Two-stage AD	Pilot-scale	Mixed	5/25	35/35	3.2	80	5.3/1.0	9/25/32	0.32/0.18/
Two-stage AD	Pilot-scale	Mixed	3/17	35/35	3.2	80	8.7/1.3	13/22/32	0.41/0.23/
Two-stage AD	Pilot-scale	Mixed	2/10	35/35	3.2	80	12.8/2.2	13/26/36	0.55/0.39/
Single-stage TAD	Full-scale	Mixed	25	51	4.6	82	1.51	64	0.86
Single-stage TAD	Full-scale	Mixed	20	55	5	81	2.0	58	1.05
Single-stage MAD	Pilot-scale	WAS	20	37	5.8	80	2.2	36	0.7
Single-stage TAD	Pilot-scale	WAS	20	55	5.8	80	2.3	48	1
Two-stage AD	Pilot-scale	WAS	2/ 8	70/55	5.8	80	15.0 / 2.3	55	1.1
Single-stage MAD	Lab-scale	Mixed	30	37	3.5-11	71-59	1.8	69	1.21

System configuration	Scale	Sludge feed type	SRT, days	T ºC	TS %	VS/TS %	OLR, Kg VS $m^{-3}d^{-1}$	VSR %	BPR L L^{-1} d^{-1}
Single-stage MAD	Lab-scale	Mixed	20	37	3.5-12	71-59	1.7	64	1.02
Single-stage MAD	Lab-scale	Mixed	10	37	3.5-13	71-59	4.1	51	1.94
Single-stage TAD	Lab-scale	Mixed	20	55	3.5-14	71-59	2.6	67	1.49
Single-stage TAD	Lab-scale	Mixed	10	55	3.5-15	71-59	3.4	61	2.02
Single-stage TAD	Lab-scale	Mixed	6	55	3.5-16	71-59	9.2	56	3.73
Single-stage MAD	Pilot-scale	WAS	17	35	2.3	68	0.9	24	0.25
Two-stage AD	Pilot-scale	WAS	2 / 15	47/35	2.3	68	7.6 / 0.9	10/16/26	0.49/0.22/
Two-stage AD	Pilot-scale	WAS	2 / 15	54/35	2.3	68	7.6 / 0.9	8/19/26	0.29/0.24/
Two-stage AD	Pilot-scale	WAS	2 / 15	60/35	2.3	68	7.6 / 0.9	67/28/35	0.03/0.35/
Single-stage MAD	Full-scale	WAS	22	36	2.70	67	1.0	15	0.15
Single-stage MAD	Full-scale	WAS	21	38	3.8	58	1.0	22	0.18
Single-stage MAD	Full-scale	WAS	33	35	3.5	63	0.8	27	0.08
Single-stage MAD	Full-scale	WAS	20	36	2.6	69	0.8	17	0.07
Single-stage MAD	Full-scale	WAS	40	34	3.9	64	0.7	13	0.04
Single-stage MAD	Full-scale	WAS	20	37	3.8	52	1.0	23	0.24
Single-stage MAD	Lab-scale	Mixed	15	37	3 - 5	75 - 80	-	50	-
Two-stage AD	Lab-scale	Mixed	1 / 14	62/37	3 - 5	75 - 80	-	61	-
Two-stage AD	Pilot-scale	Mixed	-	55/35	3	-	14.5/2.4	62	-
Two-stage AD	Full-scale	Mixed	5.5 / 21	55/37	6	60	6.5 / 1.7	60	0.60
Two-stage AD	Full-scale	Mixed	3 / 15	55/37	5.5-6.5	65	13.3 / 2.7	60	0.63
Two-stage AD	Full-scale	Mixed	2.6 / 19	55/37	3.5-5.0	60	9.7 / 1.3	60	0.30

System configuration	Scale	Sludge feed type	SRT, days	T ºC	TS %	VS/TS %	OLR, Kg VS $m^{-3}d^{-1}$	VSR %	BPR L L^{-1} d^{-1}
Two-stage AD	Full-scale	Mixed	4 / 14	55/37	2.5-3.5	75	5.6 / 1.6	55	0.67
Two-stage AD	Full-scale	Mixed	3.5 / 17	55/37	4.9	67	9.4 / 1.9	54	0.67
Two-stage AD	Full-scale	Mixed	4 / 55	55/37	1	75	1.9 / 0.1	47	0.15
Two-stage AD	Full-scale	WAS	2 / 17	36/36	5.4 VS		28.5 / 3.9	21/ 39/60	1.11/1.42/
Two-stage AD	Pilot-scale	WAS	3 / 9	37/37	6.5-7.6		18.9 / 6.2	51/42/71	1.26/2.53/
Single-stage MAD	Lab-scale	Mixed	15	35	7.0		2.00	38	0.44
Single-stage TAD	Lab-scale	Mixed	15	55	7.0		2.11	37	0.59
Two-stage AD	Lab-scale	Mixed	2 / 13	35/35	7.0		1.94	37	0.78
Two-stage AD	Lab-scale	Mixed	2 / 13	35/55	7.0		2.14	28.0	0.646

9. Observed removal efficiency of the selected 27 PhACs by different wastewater treatment technologies

The term *removal* of PhACs in WWTPs accounts for all the losses of a parent compound produced by different physico-chemical and biological mechanisms (sorption to solid matter, volatilisation and biodegradation/biotransformation). The removal by volatilisation is considered residual due to the low vapour pressures ranging from 1.0E-15 to 1.0E-7 (Table I-3). The PhACs removal extent during wastewater treatment process is influenced by many factors such as physico-chemical and biological properties of the compound, operational parameters, treatment technology used, and biomass characteristics. For this reason, a firm conclusion about the RE of each compound cannot be easily drawn, as high variation in RE rates is generally found in different WWTPs (Grandclément et al., 2017; Luo et al., 2014).

The removal rate by preliminary and primary treatments is generally considered quite poor according to many authors (Ashfaq et al., 2017; Carballa et al., 2005; Verlicchi et al., 2012; Watkinson et al., 2007; Yan et al., 2014b), and in some cases, parent compounds may even be increased in the water phase during the process, probably caused for transformation/deconjugation of undetected PhACs into the parent compounds (Ashfaq et al., 2017; Carballa et al., 2004; Göbel et al., 2005; Zorita et al., 2009). Nevertheless, Petrie et al. (2014) obtained removal efficiencies from aqueous phase from 24 to 55% for naproxen, acetaminophen and ibuprofen during primary sedimentation. Similarly, Zorita et al. (2009) obtained a load reduction in primary sedimentation between 17, 22 and 57% for ibuprofen, naproxen and diclofenac respectively, and Stasinakis et al. (2013) obtained almost 13% removal via primary sludge for diclofenac. Even though they stated that the majority of PhACs removal was achieved during secondary treatment, considerable amount could be removed for some PhACs via primary sludge.

In general, many authors concluded through a complete mass balance calculation (aqueous and suspended phase) that the removal of the majority PhACs are mainly attributed to the biodegradation/biotransformation process in the secondary biological treatment (Ashfaq et al., 2017; Gao et al., 2012c; Jelić et al., 2012; Verlicchi et al., 2012; Y. Wang et al., 2018; Xue et al., 2010; Yan et al., 2014b; Zorita et al., 2009). However, other authors highlight the importance of sorption removal for several PhACs such as some antibiotics and fluoroquinolones (Guerra et al., 2014; Jia et al., 2012; Martínez-Alcalá et al., 2017; Petrie et al., 2014; Yan et al., 2014b) as well as, fenofibrate, diazepam,

clarithromycin and hydrochlorothiazide (Jelić et al., 2011). In this sense, removal by sorption onto activated sludge flocs in water line could be a significant removal pathway for compounds with high hydrophobicity represented by the coefficient octanol-water (K_{ow}) and more specific by the sorption potential indicated by the experimental solid-water distribution coefficient (Kd) (Table I-3). To date, a simple rule has been widely accepted, if the compound has a high sorption potential (K_d > 500 L.Kg^{-1} or log K_{ow}>2.5) the PhACs tend to adsorb onto sludge and particles, being a candidate to be removed via excess sludge (RE>20%) (Luo et al., 2014; Ternes and Joss, 2015; Tran et al., 2018; Verlicchi et al., 2012).

During the biological secondary treatment, the microorganisms of the activated sludge can biodegrade the organic compounds by anabolic or co-metabolic mechanisms. According to some authors, biodegradation of PhACs is mainly attributed to co-metabolic mechanisms due to the low concentration to support substantial biomass growth (Fernandez-Fontaina et al., 2012; Fischer and Majewsky, 2014; Grandclément et al., 2017; Tran et al., 2013). Many studies have emphasized that biodegradation processes are correlated to the concentration, composition and characteristic of biomass (i.e. microbial community composition), which in turn is related to the configuration plant and operational/environmental parameters of the WWTP (such as, SRT, HRT, F/M ratio, temperature, etc.) (Grandclément et al., 2017; Tran et al., 2013; Verlicchi et al., 2012).

Figure I-11, Figure I-12 and Figure I-13 show the observed RE of the selected 27 PhACs reported in bibliography from the aqueous phase achieved after secondary biological treatment in full or pilot scale plants operating with CAS process, MBR process and all types of MBBR process, respectively. The compounds are grouped in alphabetic order in their therapeutic classes, with the mean value shown in brackets in the X-axis after the name of each compound. The numbers of papers and references revised for each compound are shown in Tables I-S4, I-S5 and I-S6. In some cases where the RE is not reported, the RE is calculated by means of eq. I.1:

$$RE\ (\%) = \frac{C_{inf} - C_{eff}}{C_{inf}} x100 \qquad \text{(eq. I.1)}$$

C is the average concentration measured in the raw influent (C_{inf}) and in the secondary effluent (C_{eff}). In the Tables I-S4 and I-S5 is possible to identify the RE provide by the authors and those calculated by means of eq. I.1. The RE values reported take into consideration the removal efficiencies by all mechanisms during the preliminary, primary

and secondary treatments: sorption onto solid matter and sedimentation in preliminary and primary treatments and a combination of sorption onto activated sludge flocs and biodegradation/biotransformation in the secondary biological treatment.

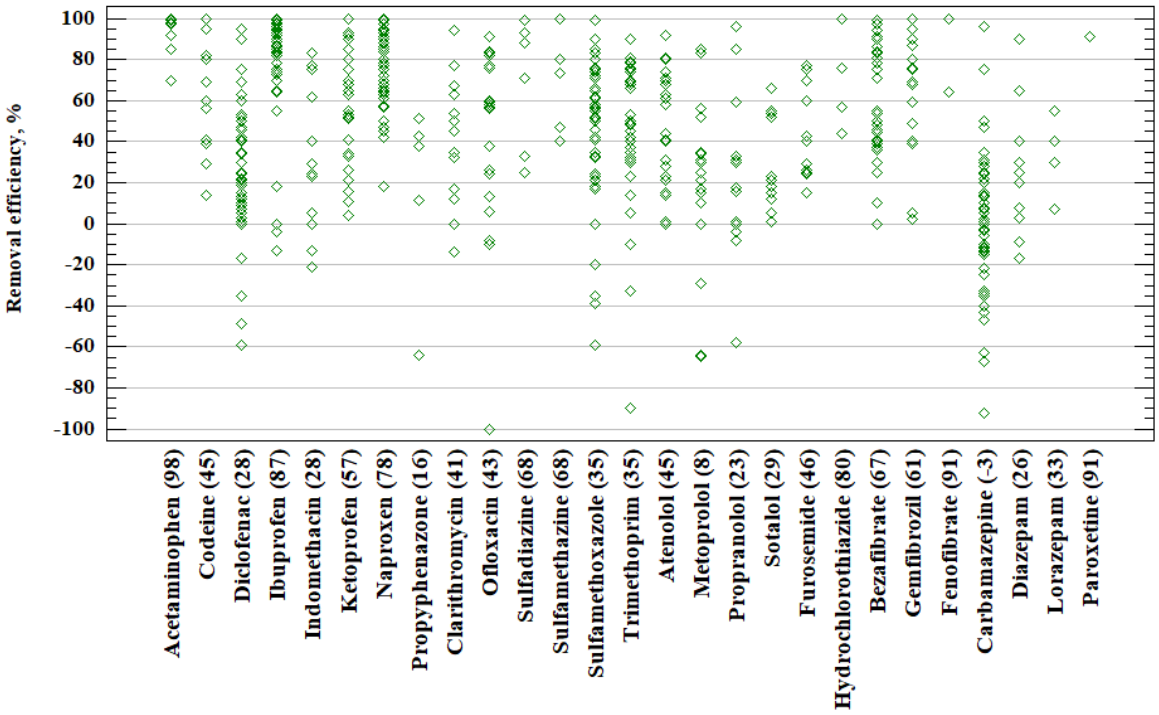
A total of 95 papers were revised, where 74 papers report RE values from full-scale CAS-WWTPs and 3 papers from pilot-scale CAS plants, 4 paper report RE values from full-scale MBR-WWTP and 18 paper from pilot-scale MBR plants, and finally, 9 papers reported RE values from pilot-scale plants operating with MBBR or IFAS process and 3 papers reported RE values from 8 advance full IFAS-WWTPs (Jewell et al., 2016; Shreve and Brennan, 2019; Yadav et al., 2019).

Referring to Figure I-11, the highest average RE values for CAS process correspond for the AIAPs acetaminophen (98%), ibuprofen (87%) and naproxen (78%), the lipid regulator fenofibrate (91%), the psychiatric medicament paroxetine (91%) and the diuretic hydrochlorothiazide (80%), highlighting that those compounds obtained the lowest RE range values. The high RE of fenofibrate and paroxetine is mainly attributed to sorption mechanisms according to Jelić et al. (2011), in accordance with the higher K_{ow} ratio (5.19) for fenofibrate and the high Kd ratio (8600-35000 L Kg^{-1}) for paroxetine reported in Table I-3. On the other hand, the lowest average RE values correspond for the psychiatric medicaments' carbamazepine (-3%) and diazepam (26%), the beta-blockers metoprolol (8%), propranolol (23%) and sotalol (29%), and the AIAPs propyphenazone (16%), diclofenac (28%) and indomethacin (28%), highlighting that those PhACs obtained the higher range values (from <-100% to 95%). The remaining PhACs obtained medium average RE values (between 30% to 75%) with a range value between 0% to 100% except for the compounds ofloxacin, sulfamethoxazole, trimethoprim and codeine with RE range values between <-100% to 100%. The high differences between RE for a given compound from one work to another are probably due to the differences in operating parameters (SRT, HRT, MLSS concentration, F/M ratio, etc.) that give different biomass composition (type of flocs and microbial community) (Grandclément et al., 2017).

Negative RE values were reported for 13 PhACs (codeine, diclofenac, ibuprofen, indomethacin, propyphenazone, clarithromycin, ofloxacin, sulfamethoxazole, trimethoprim, metoprolol, propranolol, carbamazepine and diazepam). Several reasons have been reported for the negative RE of PhACs, such as instrumental errors due to the low level of concentration found in raw influent and secondary effluent, sampling errors due to grab-sampling strategy along with the change of influent concentration during the HRT (Clara et al., 2005b; Ort et al., 2010), and transformation of conjugate forms into the

parent compounds via microbial activity or release of molecules enclosed/adsorbed in suspended particles (Ashfaq et al., 2017; Blair et al., 2015; Göbel et al., 2007).

Figure I-11. Removal efficiency (RE) of the 27 selected PhACs for the convectional activated sludge (CAS) process reported in the bibliography (Table I-S4). The corresponding mean RE values are in brackets.



Referring to Figure I-12, the highest average RE values for MBR process correspond for the AIAPs acetaminophen (97%), ibuprofen (91%), the lipid regulator bezafibrate (85%) and the antibiotics sulfadiazine (97%) and ofloxacin (84%). The AIAPs codeine (98%) and the psychiatric medicament paroxetine (90%) also have higher RE but only one data was reported. On the other hand, the lowest average RE values correspond for the psychiatric medicament carbamazepine (10%) and diazepam (26%). The remaining PhACs present medium average RE values (between 30% to 75%). Not data were found for fenofibrate and lorazepam. Only 5 PhACs obtained at least one negative RE value (trimethoprim, carbamazepine, gemfibrozil, diclofenac and naproxen) and the range values are in general lower than for CAS processes.

Referring to Figure I-13, the highest average RE values for MBRR or IFAS system correspond for the AIAPs codeine (99%), ibuprofen (96%), acetaminophen (95%), naproxen (94%) and ketoprofen (86%), and the lipid-regulator gemfibrozil (88%), highlighting that those compounds obtained low RE range values (between 60% to 100%).

On the other hand, the lowest average RE values correspond for the antibiotic's sulfamethoxazole (-3%) and sulfadiazine (0), the beta-blocker metoprolol (5%) and the psychiatric medicament carbamazepine (19%). The diuretic hydrochlorothiazide (1%) also has low average RE but only one data was found. No data were found for 10 PhACs (indomethacin, propyphenazone, ofloxacin, sotalol, furosemide, bezafibrate, fenofibrate, lorazepam, paroxetine and sulfamethazine), and 6 compounds obtained at least one negative RE in MBBR or IFAS systems (sulfadiazine, sulfamethoxazole, trimethoprim, metoprolol, propranolol and carbamazepine).

Figure I-12. Removal efficiency (RE) of the selected 27 PhACs for the membrane bioreactor (MBR) process reported in the bibliography (Table I-S5). The corresponding mean RE values are in brackets.

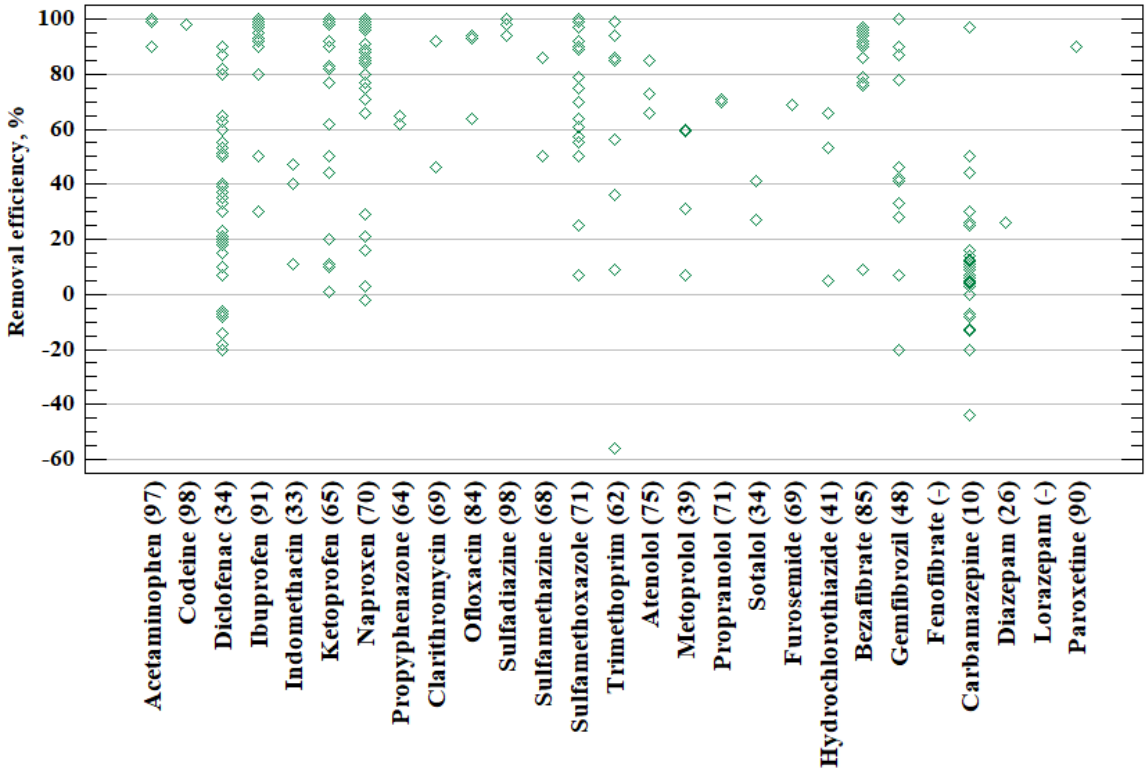
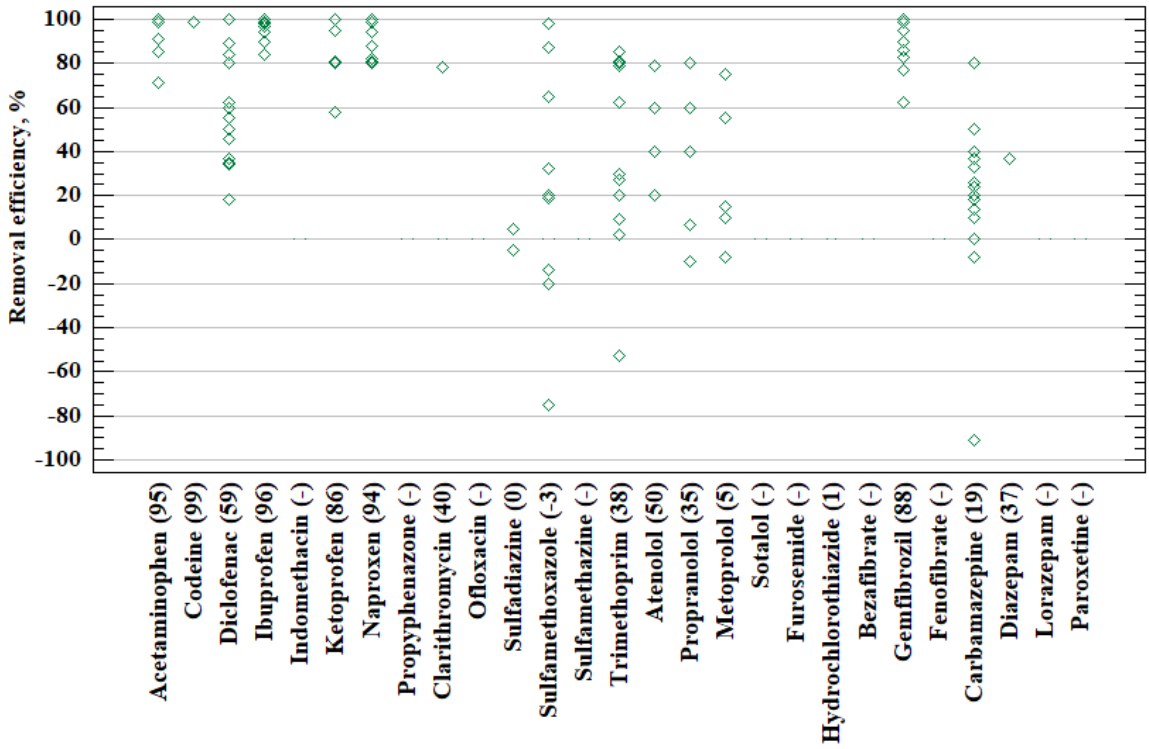


Figure I-13. Removal efficiency (RE) of the 27 selected PhACs for the integrated fixed-film activated sludge (IFAS) systems or moving bed biofilm reactor (MBBR) process reported in the bibliography (Table I-S6). The corresponding mean RE values are in brackets.



9.1. Differences in removal efficiencies of the selected PhACs between technologies

In order to find which wastewater treatment process is most efficient for PhACs removal, many authors have been comparing the two most common technology for wastewater treatment, CAS and MBR (Bernhard et al., 2006; Clara et al., 2005a, 2005b, 2004; De La Torre et al., 2015; Göbel et al., 2007; Joss et al., 2005; Kreuzinger et al., 2004; Park et al., 2017; Radjenović et al., 2009; Radjenovic et al., 2007; Sahar et al., 2011). All of them compared both technologies in parallel with the same influent, except Park et al. (2017) that compered 4 different full-scale CAS-WWTP with one MBR-WWTP.

Different conclusions and results have been reported on the removal of PhACs by MBR systems compared with CAS systems. On the one hand, several authors have been found significant better RE for some PhACs poorly removed by the CAS process, such as diclofenac (Bernhard et al., 2006; Radjenovic et al., 2007; Radjenović et al., 2009), indomethacin, propyphenazone and gemfibrozil (Radjenovic et al., 2007; Radjenović et al., 2009). However, the two pilots MBRs used by Kimura et al. (2005) obtained significantly higher RE only for ketoprofen and naproxen while comparable RE were obtained for the

other PhACs under his study. In general, Park et al. (2017), Radjenovic et al. (2007), Radjenović et al. (2009) and Sahar et al. (2011) concluded that for most of the PhACs investigated, MBR technology generally obtained higher RE than CAS process. Sahar et al. (2011) theorised that the contribution of membrane separation to higher antibiotics removal was due either to sorption to biomass or to enmeshment in the membrane biofilm. Otherwise, Park et al. (2017) and Radjenović et al. (2009) concluded that biodegradation/biotransformation is the main removal mechanisms for most PhACs, except in the case of PhACs with highly sorption characteristics.

On the other hand, many studies point out that no significant differences in RE of PhACs have been found between CAS and MBR systems when they operated at comparable operational parameters (mainly SRT and also HRT, MLSS and F/M ratio) (Clara et al., 2005b, 2005a, 2004; Kreuzinger et al., 2004); all of them highlighted that the SRT was the main operational parameter governing the RE, where a SRT_{10^oC} higher than 10 days is necessary to improve the RE. Consequently, according to Weiss and Reemtsma (2008) and Göbel et al. (2007), MBR systems could increase the RE of biodegradable trace organic compounds or PhACs because it normally operated at higher SRT (12-30 days), higher MLSS concentration (5000-2000 mg L^{-1}) and lower F/M ratio than CAS systems (Figure I-8), which may force microorganisms to biodegraded recalcitrant polar compounds due to more stronger substrate limitation at lower F/M ratio.

Due to the novelty of MBBR or IFAS process, few studies compered CAS and MBR systems with MBBR or IFAS process related to PhACs removal in the same parallel study. Falås et al. (2013), (2012) and Jewell et al. (2016) studied the RE capacity of IFAS systems during bench-scale batch experiments using activated sludge on the one hand and suspended biofilm carrier on the other hand from full-scale WWTPs. Considerable higher removal rate per unit biomass with suspended biofilm carriers were found for diclofenac by the three authors, ketoprofen and gemfibrozil by Falås et al. (2012) and trimethoprim by Falås et al. (2013). Their results and models demonstrated that in IFAS processes, attached biomass can contribute significantly to the removal of some PhACs and more research is needed to fully explore the higher RE capacity and the underlying removal mechanisms.

In this sense, De La Torre et al. (2015) realized a parallel study to compare the RE of PhACs in a semi-real plant operating with different configuration (MBR, pure MBBR-MBR, IFAS-MBR systems and full-scale CAS plant) and Luo et al. (2015) compared in a parallel study conventional MBR process with IFAS-MBR process. Both of them

concluded that the IFAS-MBR system shown similar or better RE for most of the studied PhACs, highlighting that biodegradation/biotransformation served as the primary pathway for PhACs removal (Luo et al., 2015). Additionally, the operating condition was proved to be important because the IFAS-MBR operating at low SRT and low MLSS concentration obtained only medium removal rates and the pure MBBR-MBR showed the worst performance of all membrane processes related to the lower operating HRT and lower biomass content (MLSS concentration) (De La Torre et al., 2015). Moreover, Yadav et al. (2019) evaluated the removal of drugs of addiction (included codeine) in several full-scale WWTPs with different technologies and Shreve and Brennan (2019) evaluated the RE of PhACs in six full-scale IFAS-WWTPs. Both authors concluded that hybrid MBBR systems are very effective in organic micro-pollutants removal compared with CAS-WWTPs as a consequence of higher biodegradation due to the combination of both quick and slow-growth microorganisms and higher biomass concentration (MLSS).

Finally, Escolà Casas et al. (2015), Casas et al. (2015) and Ooi et al. (2018) evaluated the RE of PhACs treating hospital wastewater by a pilot plant consisting in a series of pure MBBR reactors or combination of activated sludge, IFAS and polishing MBBR reactors. They concluded that MBBR and IFAS technology reach similar or better RE of PhACs compared to other technologies, where the biodegradation generally occurred in parallel to the removal of COD and nitrogen, indicating co-metabolism (Casas et al., 2015; Ooi et al., 2018). Moreover, the biomass in the final IFAS reactors low-loaded is adapted for growing on hardly degradable organic matter (Escolà Casas et al., 2015). They stated that the MBBR and IFAS system can be seen as a promising solution for hospital wastewater treatment.

In order to find significant differences in removal rate values reported by bibliography between each technology (CAS, MBR and MBBR or IFAS) for the 27 selected PhACs, the average, standard deviation and number of data are shown in Table I-9. Compound marked with an asterisk ($p<0.05$*) were significantly different between the three technologies according to the Kruskal-Wallis test. Significantly higher RE were found in the MBBR or IFAS system compared with both CAS and MBR processes for the recognised biodegradable compound ketoprofen (86%), naproxen (94%) and gemfibrozil (85%) and the considered recalcitrant compound diclofenac (59%) and carbamazepine (19%). Statistically similar RE values were found for acetaminophen, ibuprofen, clarithromycin, atenolol and metoprolol and significantly higher RE were found for the antibiotics ofloxacin, sulfamethoxazole and trimethoprim, and the lipid-regulator bezafibrate for the MBR process compared with CAS systems and MBBR systems (only

sulfamethoxazole and trimethoprim). No enough data were found for the rest of PhACs to draw a firm conclusion.

Table I-9. Average removal efficiencies (RE, %) of the selected 27 PhACs for three wastewater treatment systems (CAS, MBR and MBBR or IFFAS). It is evaluated from the data set obtained from the revised bibliography (Table I-S4, I-S5, I-S6). Values shown are means ± SD; the number of data used is shown in brackets. Compound marked with asterisk ($p < 0.05$*) were significantly different between the three technologies, according to the Kruskal-Wallis test.

Groups	Pharmaceutical compounds	RE CAS	RE MBR	RE MBBR
AIAPs	Acetaminophen	98 ± 6 (26)	97 ± 4 (12)	95 ± 10 (10)
	Codeine	45 ± 61 (12)	98 (1)	99 (1)
	Diclofenac*	**28 ± 36 (58)**	**34 ± 29 (40)**	**59 ± 26 (17)**
	Ibuprofen	87 ± 23 (76)	91 ± 16 (32)	96 ± 5 (10)
	Indomethacin	28 ± 33 (12)	33 ± 19 (3)	0
	Ketoprofen*	**57 ± 27 (32)**	**65 ± 35 (17)**	**86 ± 16 (6)**
	Naproxen*	**78 ± 18 (52)**	**70 ± 33 (22)**	**94 ± 8 (14)**
	Propyphenazone	16 ± 47 (5)	64 ± 2 (2)	
Antibiotics	Clarithromycin	41 ± 31 (13)	69 ± 33 (2)	40 ± 54 (2)
	Ofloxacin*	**43 ± 46 (20)**	**84 ± 17 (3)**	
	Sulfadiazine	68 ± 32 (6)	98 ± 3 (4)	0 ± (2)
	Sulfamethazine	68 ± 25 (5)	68 ± 25 (2)	0
	Sulfamethoxazole*	**35 ± 64 (45)**	**71 ± 25 (21)**	**-3 ± 73 (12)**
	Trimethoprim*	**35 ± 46 (34)**	**62 ± 49 (11)**	**38 ± 44 (11)**
Beta-blocker	Atenolol	45 ± 28 (22)	75 ± 10 (3)	50 ± 25 (4)
	Metoprolol	8 ± 62 (19)	39 ± 25 (4)	5 ± 68 (6)
	Propranolol	23 ± 41 (13)	71 ± 1 (2)	35 ± 37 (5)
	Sotalol	29 ± 23 (11)	34 ± 10 (2)	
Diuretics	Furosemide	46 ± 22 (13)	69 ± (1)	
	Hydrochlorothiazide	80 ± 25 (6)	41 ± 32 (3)	
Lipid-regulators	Bezafibrate*	**67 ± 30 (41)**	**85 ± 20 (20)**	
	Fenofibrate	91 ± 18 (4)		
	Gemfibrozil	**61 ± 30 (16)**	**48 ± 37 (11)**	**88 ± 11 (11)**
Psychiatrics medications	Carbamazepine*	**-3 ± 40 (62)**	**10 ± 23 (33)**	**19 ± 37 (15)**
	Diazepam	26 ± 31 (11)	26 ± (1)	25 ± (1)
	Lorazepam	33 ± (4)		
	Paroxetine	91 ± (1)	90 ± (1)	

To conclude, no significant difference exists between CAS and MBR treatments, highlighting that the observed differences in RE for a given compound between each technology and from one work to another are more probably due to the differences in the main operational parameters such as SRT, HRT, MLSS concentration, redox condition and F/M ratio of each treatment process that produced different biomass composition (a type of flocs and microbial community) (Alvarino et al., 2018a; Grandclément et al., 2017; Weiss and Reemtsma, 2008). However, recent studies suggested improvements in PhACs

biodegradation/biotransformation using hybrid MBBR technology due to the combination of suspended activated sludge and attached-growth biomass that gives a combination of slow-growing microorganisms in the carrier media and quick-growing microorganisms in the activated sludge, which increases the range of possible active strains and microbial community (Casas et al., 2015; Falås et al., 2013; Grandclément et al., 2017; Leyva-Díaz et al., 2020; Polesel et al., 2017; Torresi et al., 2016). Finally, due to the affordable cost and well-demonstrated high efficiency in organic matter, nutrients and micropollutants removal, hybrid MBBR systems became one of the most promising technologies for CAS-WWTP upgrading.

9.2. Influence of operating condition in removal efficiency of PhACs

A bibliography review is done to find the effects of the main operational parameter in a WWTP governing the removal efficiency of PhACs: SRT, biomass concentration, F/M ratio, HRT, as well as redox condition.

9.2.1. Effect of sludge retention time, biomass concentration and F/M ratio

The sludge retention time, also known as sludge age or solid retention time (SRT) is considered the main design parameter of a WWTP because it is related to the growth rate of microorganisms and directly correlated to the biomass concentration which is also related to the F/M ratio. A WWTP operating at high SRT normally works at higher MLSS concentration and lower F/M ratio at the same organic loading rate, which gives more amount of biomass capable to biodegrade recalcitrant contaminants in less substrate concentration available (low F/M ratio). High SRT allows the increase of slow-growing microorganisms which increase the microbial diversity capable of biodegrading recalcitrant compounds (Grandclément et al., 2017; Helbling et al., 2012; Tran et al., 2013). For instance, it has been reported that an increase in SRT could enhance the removal of some PhACs such as diclofenac, ketoprofen, naproxen, bezafibrate, ibuprofen, sulfamethoxazole and trimethoprim. (Clara et al., 2005a, 2004; Fernandez-Fontaina et al., 2012; Kimura et al., 2007; Kreuzinger et al., 2004; Tambosi et al., 2010; Xia et al., 2012). In this sense, a minimum SRT of 10-15 days have been proposed by Clara et al. (2005a). De La Torre et al. (2015) obtained higher RE of PhACs in a pilot-scale IFAS-MBR plant operating at higher SRT (20 days vs 10 days), higher MLSS concentration and low F/M ratio. Surprisingly, RE above 80% were reported for carbamazepine using an IFAS-MBR system at 20 days of SRT. Moreover, several authors mentioned that high SRT combined

with higher MLSS concentration and lower F/M may induce microorganisms to metabolise even less biodegradable compounds (Göbel et al., 2007; Verlicchi et al., 2012; Weiss and Reemtsma, 2008).

However, not clear correlation between SRT and RE of PhACs were found in others studies in particular for beta-blockers, carbamazepine or ofloxacin (Joss et al., 2005; Vieno et al., 2007). Falås et al. (2016) similarly no found a strong correlation between RE and SRT for more than 20 micropollutants in bioreactors ranging from 25 to 80 days. Concerning carbamazepine, Bernhard et al. (2006) and Maeng et al. (2013) noticed no RE regardless of the change of SRT using MBR bioreactors. Regarding diclofenac, Clara et al. (2005b) and Suárez et al. (2012) reported higher RE at higher SRT in an MBR pilot-plant but no strong correlation where found. Besides, Bernhard et al. (2006) obtained enhance RE when the SRT was increased from 20 to 322 days using an MBR reactor, reaching 53% of RE at 322 days of SRT.

To concluded, a Spearman´s rank analysis where done between SRT and RE using pair values obtained from studies that report both data (Table I-S8, CAS and MBR system where included). From the 27 selected PhACs only 6 obtained significant statistical Spearman rank correlation ($p<0.05$): bezafibrate ($\rho=0.70$; $p=0.000$), diclofenac ($\rho=0.47$; $p=0.0001$), gemfibrozil ($\rho=0.64$; $p=0.04$), ibuprofen ($\rho=0.46$; $p=0.0001$), ketoprofen ($\rho=0.76$; $p=0.0017$) and trimethoprim ($\rho=0.48$; $p=0.03$). These correlation are well documented in different studies for these compounds (Bernhard et al., 2006; Clara et al., 2005a, 2004; Göbel et al., 2007; Kimura et al., 2005; Kreuzinger et al., 2004).

9.2.2. Effect of hydraulic retention time

The HRT is the time that the liquid phase stays in the bioreactor, therefore it corresponds to the reaction time for PhACs linked to the aqueous phase. The HRT is inversely related to the F/M ratio at the same MLSS and organic matter concentration. The influence on biodegradation/biotransformation efficiencies of different PhACs was largely investigated. Bernhard et al. (2006) found no significant correlation between RE and HRT in a MBR reactor, even higher REs were found at 7 hours of HRT in the MBR reactor compared with 22 hours in the reference CAS-WWTP, nevertheless MBR reactor at 7 hours reached higher MLSS concentration and higher SRT. (Vieno et al., 2007) also reported no clear relationship between RE of PhACs and HRT from 12 different CAS-WWTPs in Finland, having HRT between 1.5 to 20 hours.

On the other hand, Gros et al. (2010) obtained in seven CAS-WWTP the RE of several PhACs and the corresponding half-live times, assuming that compound degradation followed pseudo-first order kinetics, also previously observed by Joss et al. (2006). They suggested that higher HRT is required in WWTPs due to the high half-lives observed for the majority of PhACs, and concluded that HRT is a key parameter regarding pharmaceutical elimination, besides other operational parameters. They proposed a simple rule: PhACs that have high biodegradable rate loss constant (K_{biol}), high half-live and low K_d values, are more influenced by HRT, whereas compounds with high K_d values and low K_{biol} (low biodegradation rate) are more influenced by SRT.

A Spearman´s rank analysis was done between HRT and RE using pair values obtained from studies those report both data (Table I-S9). From the 27 selected PhACs only the highly biodegradable PhACs bezafibrate and naproxen obtained significant statistical Spearman rank correlation ($p<0.05$).

9.2.3. Effect of redox condition

Since the biological nutrient removal process in CAS-WWTPs were developed (Figure I-8, C and D), the existence of anaerobic, anoxic and aerobic condition occurred in the same bioreactors. Different metabolites, bioreactions and removal mechanism of various pollutants occurred under these different redox conditions (Alvarino et al., 2016, 2018a; Grandclément et al., 2017). For instance, high RE of PhACs have been found at high levels of nitrogen removal (anoxic-aerobic condition) (Clara et al., 2005a; Marx et al., 2015; Miège et al., 2009; Thiebault et al., 2017; Vieno et al., 2007). Vieno et al. (2007) reported higher RE of atenolol and sotalol in WWTP with higher nitrogen removal rate, this assumption was verified by Thiebault et al. (2017) when a statistically significant correlation between RE and total nitrogen removal was found for atenolol. Moreover, out of the 15 PhACs investigated by Marx et al. (2015), the RE of clindamycin and ciprofloxacin show a significant correlation to nitrogen removal, as well as, SRT, HRT and temperature. In this sense, Miège et al. (2009) concluded from a literature review of various WWTPs for a limited number of PhACs that activated sludge with nitrogen removal and MBR process were the most efficient ones.

In this context, Suarez et al. (2010) studied the RE in aerobic and anoxic conditions. They observed an increase of diclofenac RE from 0% to 74% in an aerobic reactor due to the development of nitrifying biomass, while for ibuprofen higher REs in aerobic (95%) and anoxic (75%) reactors after an acclimation period were obtained. They divided into

three groups PhACs concerning their potential to be removed in different redox condition: highly biodegradable in both condition (aerobic and anoxic) such as ibuprofen, no removal in an anoxic condition such as diclofenac and naproxen and finally recalcitrant to both conditions such as sulfamethoxazole, trimethoprim, carbamazepine and diazepam. Otherwise, Lahti and Oikari (2011) compared the RE of diclofenac and naproxen under both aerobic and anaerobic condition, where naproxen was biodegraded in both condition and diclofenac was recalcitrant. In this sense, Alvarino et al. (2018b) also reported that naproxen is well removed in both conditions (aerobic and anaerobic) and concluded that aerobic systems are more efficient for the majority of PhACs, although anaerobic units can improve the biodegradation/biotransformation of some PhACs such as naproxen, sulfamethoxazole and trimethoprim.

In general, these results showed that certain improvements in biodegradation/biotransformation of some PhACs can be achieved by combining different redox conditions that results in an increased microbial diversity and a broader enzyme spectrum (Alvarino et al., 2018b; Falås et al., 2016; Grandclément et al., 2017; Suárez et al., 2012; Wolff et al., 2018).

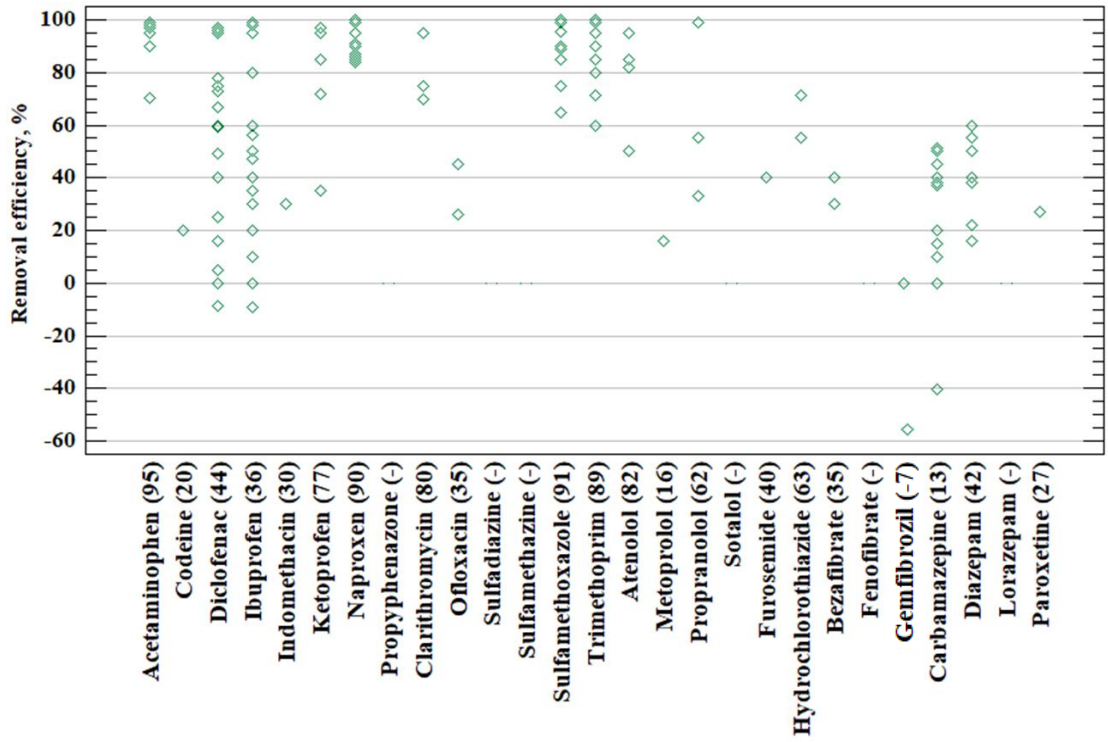
10. Observed removal efficiency of the selected 27 PhACs by anaerobic digestion of sewage sludge

Figure I-14 shows the RE of the selected 27 PhACs reported in bibliography for the anaerobic process of sewage sludge from lab/pilot-scale plants and full-scale AD plants. The compounds are grouped in alphabetic order into their therapeutic classes, with the mean value shown in brackets in the X-axis after the name of each compound. The numbers of papers and references revised for each compound are shown in Table I-S7. The RE reported from Martín et al. (2012b) and Radjenović et al. (2009) was calculated by means of eq. I.1. The losses of the parent compound by the AD process correspond mainly to the biodegradation/biotransformation process because the RE is done by mass balance analysis and no separation process occurred between influent and effluent samples.

A total of 16 papers were revised, where only 5 papers reported data from full-scale AD plants and 11 reported data from lab/pilot scale AD plants operating at different SRT and temperature (mesophilic and thermophilic range). Only Samaras et al. (2014) studied the RE of PhACs comparing in parallel mesophilic, thermophilic and two-stage AD systems.

The most anaerobically biodegradable compounds with the highest average RE values correspond for the AIAPs acetaminophen (95%) and naproxen (90%) and the antibiotics sulfamethoxazole (91%) and trimethoprim (89%), highlighting that those compounds obtained very low RE range values (between 60 to 100%) with the high number of data (>10). On the other hand, the less biodegradable compounds in AD process with the lowest average RE correspond for the lipid-regulator gemfibrozil (-7) and the psychiatric medicament carbamazepine (13%), also metoprolol (16%) and codeine (20%) showed low RE but only one data were found in the bibliography. RE values for AD process were no reported for 6 PhACs (fenofibrate, lorazepam, propyphenazone, sotalol, sulfadiazine and sulfamethazine). The remaining PhACs obtained medium average RE values (between 27% to 82%) and negative RE was reported in at least one study for gemfibrozil, carbamazepine, ibuprofen and diclofenac.

Figure I-14. Removal efficiency (RE) of the selected PhACs for the anaerobic digestion process reported in the bibliography (Table I-S7). The corresponding mean RE values are in brackets.



The differences between RE reported from one work to another are less noticeable in sewage sludge AD process that for the wastewater treatment process, except for the AIAPs diclofenac and ibuprofen. Concerning to diclofenac, very high REs (>95%) were reported by Samaras et al. (2014) and high RE (60%-78%) after sludge adaptation was reported by

Carballa et al. (2007b), (2007a), (2006) and Zhou et al. (2017), surprisingly, these are lab-scale experiments that used spiked PhACs into the feed to obtain a sufficiently high concentration. However, low-medium RE (between 16% and 60%) were reported for diclofenac by Gonzalez-Gil et al. (2019), Jelić et al. (2012), Malmborg and Magnér (2015), Narumiya et al. (2013) and Yang et al. (2017) and no removal were reported for Carballa et al. (2007b), Phan et al. (2018) Radjenović et al. (2009) and Yang et al. (2016), where only Carballa et al. (2007b) and Malmborg and Magnér (2015) used spiked PhACs. Regarding ibuprofen, very high and high RE (>80%) were reported by Samaras et al. (2014) and (2013), low-medium RE (between 20% and 60%) were reported by Carballa et al. (2007b), (2007a), (2006), Gonzalez-Gil et al. (2016), Malmborg and Magnér (2015) and Martín et al. (2012b), while no removal was reported by Phan et al. (2018), Radjenović et al. (2009) and Yang et al. (2017), (2016). Normally, higher RE for ibuprofen was reported by lab-scale experiment with used spike PhACs. In this sense, Gonzalez-Gil et al. (2016) and Phan et al. (2018) point out that striking differences in RE between studies can be due to the use of spike PhACs, particularly for persistent PhACs in AD process (e.g. diclofenac, ibuprofen and carbamazepine). In fact, it has been proved by Dictor et al. (2003) that the compounds freshly added are more bioavailable and biodegradable than aged compounds.

Many authors have been comparing the RE of PhACs in different digesters operating at different temperature (mesophilic and thermophilic range), different SRT and OLR. In general, the majority of the studies (Carballa et al., 2007b, 2007a, 2006; Gonzalez-Gil et al., 2016; Malmborg and Magnér, 2015; Yang et al., 2017, 2016) did not found a significant correlation between the RE and operational parameters (T, SRT and OLR). Otherwise, Carballa et al. (2006) have found influent of temperature, SRT and pretreatment only in the RE of ibuprofen and roxithromycin and Zhou et al. (2017) concluded that SRT played an important role on the RE of caffeine, diclofenac and triclosan not only during AD but also during AD combined with enhancing treatment. The relative independence between operational parameters and RE of PhACs found in bibliography could be related the variation in feed concentration that masks the improvement of RE when SRT was increased as well as the PhACs are no the main substrate for AD process (Yang et al., 2016).

Phan et al. (2018) and Malmborg and Magnér (2015) reported a significant correlation between lipophilicity and persistence of PhACs during AD process, while Yang et al. (2016) showed that the overall PhACs removal by AD process was not significantly influenced by their hydrophobicity.

Nowadays, the relationship between PhACs biotransformation/biodegradation and the microbial activity in AD process is still poorly investigated. Stasinakis (2012) indicated that abundance of PhACs degrading microorganisms is a crucial factor for their removal in AD process due to the favored biodegradation of diclofenac, diazepam and estrogens by biomass acclimatization (Carballa et al., 2007b, 2006) and Gonzalez-Gil et al. (2018) suggest by their results that the methanogenesis step plays an important role in the overall PhACs removal during AD process. This conclusion agrees with Ghattas et al. (2017) that reported a wide variety of organic micro-pollutants bioreactions by methanogens microorganisms. Concerning to the two-stage AD systems only Samaras et al. (2014) reported slightly better RE of endocrine disrupting compounds, whereas did no found differences in the RE of AIAPs.

11. Study of microbial community diversity in wastewater treatment and sewage sludge treatment

During many decades, the design, operation and development of wastewater treatment and sewage sludge treatment processes have been done mainly based on physico-chemical parameters information with scarce knowledge about the microbial community and metabolic potential (Gilbride et al., 2006). The biological fundament of wastewater bioreactors and sludge digester was viewed as “black boxes” and those bioreactors challenged with random stability and performance breakdowns such as bulking or foaming problems (Madoni et al., 2000; Maza-Márquez et al., 2016a), nitrification failure in wastewater bioreactors (Gilbride et al., 2006; Xia et al., 2018) or foaming problems, low start-ups and unstable fermentation in sludge digesters (Ghosh et al., 1995; Leite et al., 2016). The knowledge on microbial community structure and functions by the culture-independent molecular methods developed since the 1990s was crucial for the optimization and development of these bioreactors by engineers that helped to solve the mentioned problems (Sanz and Köchling, 2007; Xia et al., 2018).

Conventional microbiological methods, based in culture-dependent techniques have provided extensive information on the microbial community and help us to identified key microbial population with specific metabolic processes in anaerobic digester and wastewater bioreactors (Sanz and Köchling, 2007; Vanwonterghem et al., 2014). However, the majority of microorganism could not be cultivated, where most culture media tend to favour the growth of specific groups of microorganisms, whereas other important groups do not proliferate. Despite the efforts to simulate the real condition in the culture media, it

was difficult to recreate the nutritional requirements, the physical-chemical conditions and the complex symbiotic relationships between the microorganisms that occur under natural conditions (Sanz and Köchling, 2007; Vanwonterghem et al., 2014).

Over the last two decades, the application of culture-independent molecular methods to wastewater bioreactors and anaerobic digesters together with other complementary techniques such as digital imagen (FISH) and the *-omics* revolution occurred during the last decade, has enhanced our knowledge into the community composition and function of dominant populations, as well as, our understanding of how reactors configuration, feedstock, operational conditions and parameters influence the microbial community structure and dynamics (Ferrera and Sánchez, 2016; Vanwonterghem et al., 2014).

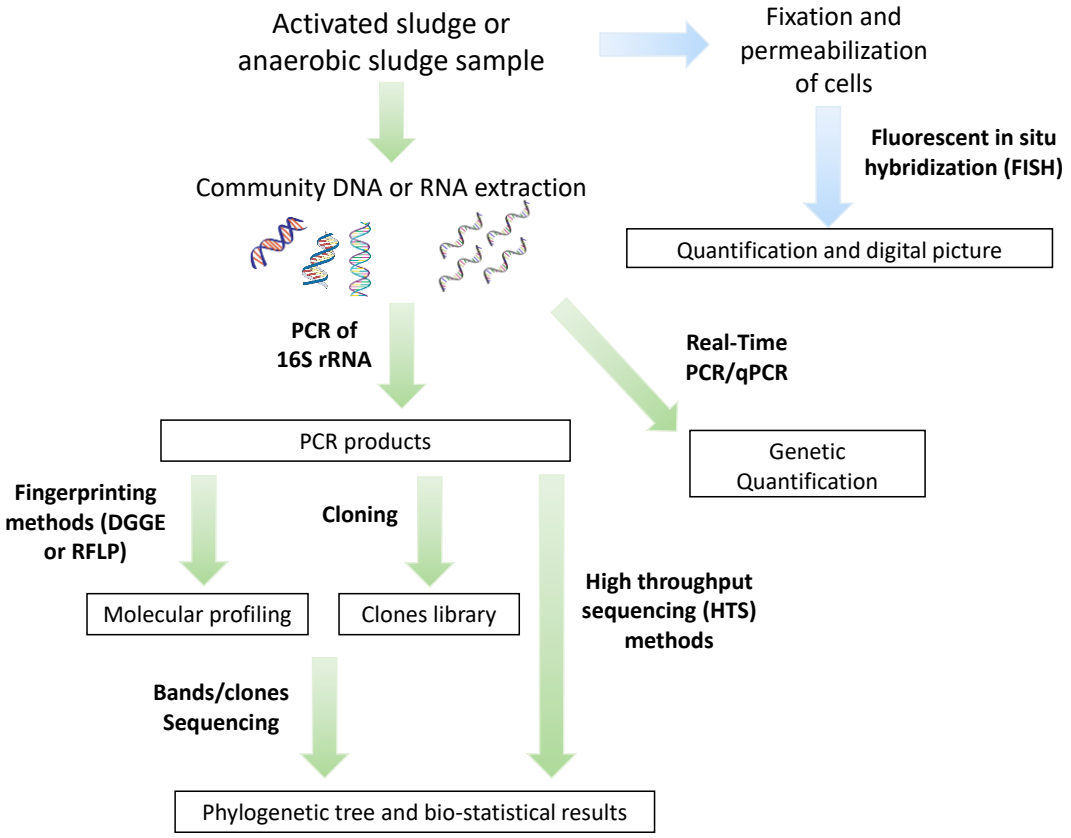
11.1. Culture-independent methods to study the microbial community of activated sludge and anaerobic sludge

Figure I-15 shows in a schematic diagram the main independent-culture techniques used nowadays to study the microbial community structure and diversity of wastewater bioreactors and anaerobic sludge digesters. The Polymerase Chain Reaction (PCR) technique developed by Kary Banks Mullis in 1983 together with the application of traditional fingerprinting methods (e.g. denaturing/temperature gradient electrophoresis "DGGE" and terminal restriction fragment length poly-morphism "T-RFLP") and cloning using the 16S rRNA gene revolutionized the microbial ecology research. These PCR-approaches have been used for decades to study the diversity and presence of microorganisms in different environments, allowing the identification of the key microorganisms involved in different processes (acidogenesis, methanogenesis, ammonia oxidation, carbon-degrading, etc.) into the anaerobic digesters and wastewater bioreactors (Ferrera and Sánchez, 2016; Vanwonterghem et al., 2014). The fluorescent *in situ* hybridization with DNA probes (FISH) also made possible, during the last decades, the quantification and identification of the dominant groups involve in different environments, while in recent years, the application of real-time quantitative PCR (qPCR) has become the main molecular method for the absolute quantification of prokaryotic and eukaryotic microorganisms in a wide range of engineered and natural environments (Maza-Márquez et al., 2020; Sanz and Köchling, 2007). However, these techniques (mainly cloning and sequencing) are high-costing, time-consuming and have relatively low throughput compared with the high-throughput sequencing (HTS) methods that have strongly

decreased the time and cost, and increase the yield of sequence data generated (Ferrera and Sánchez, 2016).

The application of the HTS techniques (e.g., 454-pyrosequencing and Illumina sequencing platform among others) to 16S rRNA gene amplicon sequencing accelerated the metagenomic studies (the massive DNA sequencing of all genes present in a microbial community) in wastewater and sewage sludge treatment process. The ability to produce millions of sequencings reads at low cost allows the simultaneous examination of spatially and temporally samples, which provides large amount of data for correlation analyses. Important correlation analyses between community composition and environmental/operational condition such as influent/effluent concentration of organic matter and nutrients, temperature, oxygen dissolve, SRT, F/M ratio, HRT, etc. have been reported in many studies nowadays (Castellano-Hinojosa et al., 2018a; Chen et al., 2017; Ferrera and Sánchez, 2016; Maza-Márquez et al., 2015; Muñoz-Palazon et al., 2018; Reboleiro-Rivas et al., 2016; Rodriguez-Sanchez et al., 2016; Tian and Wang, 2020; Vanwonterghem et al., 2014; Xia et al., 2018). In summary, metagenomics studies based on HTS methods complemented with qPCR and traditional methods are powerful tools in profiling genomic structures, diversity and functional potentials of the microbial community members of wastewater or sewage sludge bioreactors (Xia et al., 2018).

Figure I-15. Schematic diagram displaying the main independent-culture techniques used to study the microbial community structure and diversity of wastewater bioreactors and anaerobic sludge digesters. The techniques are shown in bold letter and the different results/products in boxes.



11.2. Diversity and structure of microbial communities in wastewater treatment plants

The microbial communities developed in WWTPs are characterized by the presence of a wide and complex metabolic and genetic diversity, allowing the biodegradation of most organic pollutants and the continuous adaptation of microbial population to new operational and environmental conditions. These characteristics are essential in wastewater treatment plants, since they are subject to continuous fluctuations. Microbial community diversity in WWTPs reflects the richness of species in the specific system, many authors point out that high microbial diversity increase the stability of the system and influence the treatment performance (Chen et al., 2017; Xia et al., 2018). However, the "biological stability" of the wastewater treatment process not only seems to be due to the great microbial diversity and its dynamics, but also to the extensive functional redundancy that characterizes the microbial communities developed in wastewater treatment plants (Ferrera

and Sánchez, 2016; Tian and Wang, 2020). Functional redundancy occurred when different species are capable of performing the same biochemical functions, therefore the efficiency of the process does not depend on a single species or on the dominant species. This characteristic increases the robustness of the process when fluctuation occurred.

A meta-analysis of microbial community structure realized by Tian and Wang (2020) over 185 samples taken from 50 WWTPs across 13 countries yielded a total of 69 Phyla, 230 class, 452 orders, 738 families and 1382 genera, only a small percentage of OTUs (9.01%) were not classified to any taxon. The predominant phyla were *Proteobacteria* (50.70% of relative abundance), *Bacteroidetes* (20.78%), *Chloroflexi* (5.56%), *Actinobacteria* (4.99%) and *Firmicutes* (3.99%). The composition of these microbial communities in CAS and MBRs systems seem to show a high degree of similarity (Tian and Wang, 2020; Xia et al., 2010; Zhang et al., 2012). However, the composition of the microbial community appears to differ between MBBR process and CAS and MBR systems. Tian and Wang (2020) found significant taxonomic difference between samples from activated sludge and biofilm, where the top 3 phyla in MBBR process were *Proteobacteria*, *Bacteroidetes* and *Firmicutes*, while the Families *Bacillaceae*, *Lactobacillales* and *Lachnospiraceae* belonging to the phylum *Firmicutes* were strictly enriched. The authors stated that unlike activate sludge processes, most of the enriched bacteria in biofilm processes were obligate or facultative anaerobe due to the existence of anaerobic layer in biofilm. Biswas and Turner (2012) also concluded that MBBR communities differ substantially from CAS due to the existence of two types of communities: the biofilm community, which seems to be dominated by anaerobic microorganisms and a suspended community that includes fast-growing aerobic bacteria.

In a review of microbial community diversity, Xia et al. (2018) highlight the presence of uncultured phyla such as TM7, OD1, OP10 and WS3 and the importance of the characterization of minor population by HTS methods such as antibiotic resistant bacteria/genes and bacterial pathogens. Apart from the overall microbial communities, many investigations have been made to find the main microbial group specialize with a specific function such as nitrifiers (ammonia oxidizing bacteria or archaea, AOB or AOA, respectively and nitrite oxidizing bacteria, NOB), denitrifiers, phosphorus accumulating organisms (PAOs) and filamentous bacteria (Ferrera and Sánchez, 2016; Xia et al., 2018).

Currently, numerous studies have indicated that process configuration, operating/environmental parameters and influent chemical composition are the main driven factors for the microbial structure change in WWTPs. However, there are scarce studies

about the influence of emerging micro-contaminants (PhACs, endocrine disrupting chemicals, etc.) on the microbial community structure and how to optimize the microbial community and maintain the stable operation process under different stress conditions and fluctuations (Chen et al., 2017).

11.3. Diversity and structure of microbial communities in anaerobic digester of sewage sludge

The microbial communities developed in anaerobic digesters are characterized by the presence of a wide and complex metabolic community of strict and facultative anaerobic members of the bacteria and archaea domains, allowing the anaerobic biodegradation of organic matters into an energetic valuable compound such as methane. The Bacteria members are the majority in terms of quantity and biodiversity compare to Archaea (Collins et al., 2006) and are the responsible for the three first metabolic processes (hydrolysis, acidogenesis and acetogenesis) previously described in the Figure I-10. However, the final methanogenesis process step is completed by the participation of Archaea domain. Although members of Archaea domain have a relatively low abundance and diversity compared to members of bacteria, they play an important role because they are the only ones responsible for methane production. Scarce information about anaerobic eukaryotic organisms is available to date. Some of the Fungi found in anaerobic environments contribute to the degradation of organic matter, so they could be implicated in the hydrolysis of organic matter in the anaerobic digestion of sewage sludge (Castellano-Hinojosa et al., 2018a).

During the last decade, the microbial diversity found in anaerobic digesters have been increasing constantly: Pervin et al. (2013) found in a sewage sludge thermophilic AD that around 90% of the obtained OTUs correspond to microorganisms not detected previously in this environment with low similarity with culture-microorganisms. Additionally, Mei et al. (2017) demonstrated by analysing the microbial communities of 90 full-scale AD of sewage sludge at 51 WWTPs from five countries that AD microbial communities have high heterogeneity, and rejected the possibility to define a single core microbiome for all digesters that differed in environmental/operational conditions. They classified the AD microbiome into eight cluster driven by operational conditions instead of geographical location, and detected that residue populations associated with the undigested feed activated sludge were commonly observed in all the AD samples. The major 15 phyla found for Mei et al. (2017) in the 90 full-scale AD where *Bacteroidetes, Thermotogae,*

Proteobacteria, Ca. Cloacimonetes, *Firmicutes*, *Spirochaetes*, OP1, Ca. Fermentibacteria, *Synergistetes*, *Chloroflexi*, *Tenericutes*, EM3, Ca. Atribacteria, Ca. Latescibacteria and *Euryarchaeota.*

The methanogenic archaea are the main phylotypes responsible for the methane production. According to the type of substrate used, they can produce it be two different routes (Figure I-10), hydrogenotrophic route (CO_2, H_2, or formic acid) or acetotrophic route (acetate, methanol and methyl amines). Most of the archaea found in AD processes are hydrogenotrophic, while only the order *Methanosarcinales* are acetotrophic (Castellano-Hinojosa et al., 2018a). Archaea belong to the phylum *Euryarchaeota*, formerly alienated into six established orders (*Methanobacteriales, Methanococcales, Methanocellales, Methanomicrobiales, Methanosarcinales, Methanopyrales*) and a proposed one (Methanomassiliicoccales) (Ferrera and Sánchez, 2016). The molecular studies on sewage sludge AD have shown that the archaeal communities are mainly composed by the orders *Methanobacteriales, Methanosarcinales* and *Methanomicrobiales* (Ferrera and Sánchez, 2016). Accordingly, the most relative abundant genus found by Mei et al. (2017) in 90 full-scale AD of sewage sludge was: *Methanothermobacter* (hydrogenotrophic methanogens belonging to *Methanobacteriales* phyla); *Methanosaeta and Methanosarcina* (acetotrophic and versatile methanogens respectively belonging to *Methanosarcinales* phyla); *Methanoculleus, Methanolinea, Methanoregula* and *Methanospirillum* (hydrogenotrophic methanogens belonging to *Methanomicrobiales* phyla) and Ca. Methanofastidiosa (hydrogenotrophic methanogens, novel candidatus archaeal clade).

During the last decade, engineers and microbial ecologist have shown increasing interest into the microbial community structure and function to improve the AD process performance. Some authors have found interesting correlation between environmental/operational parameters, performance rate (SRT or HRT, total solid/volatile influent, OLR, type of feedstock, temperature, methane production yields, etc.) and the microbial population governing the AD process (Carballa et al., 2011; De Vrieze et al., 2017; Gonzalez-Martinez et al., 2016; Hao et al., 2016; Kundu et al., 2017; Langer et al., 2019; P. Wang et al., 2018; Westerholm and Schnürer, 2019). For instance, Hao et al. (2016) realized a deep investigation into the main bacterial and archaea phylotypes involved in methane production under different operational parameters, or Kundu et al. (2017) found that the family *Methanosaetaceae* was a possible candidate for biomonitoring due to its sensitivity to fluctuations in the AD process. Those studies shown that the

biomonitoring and identification of the critical representative microorganisms by means of these molecular tools can help to improve the stability and performance of AD processes.

However, the constantly increasing knowledge about the microbial diversity, structure and function of AD process, point out the difficulty and complexity to properly characterize the system, particularly as many members of the same genus are often able to degrade different compounds and many microorganisms belong to candidate phyla and even unknown (Westerholm and Schnürer, 2019). Thus, the continues investigations with molecular (screening) tools in order to find the most effective operating parameters to achieve maximum AD performance are still quite relevant. It is important to identify and characterize the key players in the process, its function and interaction between microbial community structure and operating parameters and performance.

Supplementary material for the general introduction:

Table I-S1: Numbers of papers and references examined to find the raw urban WWTPs influent concentration and seco selected PhACs in the WWTPs studied by these papers.

Groups	Pharmaceutical compound	Numbers of papers	References for raw urban WWTP Influent	Numbers of papers	References
Analgesics/Anti-inflammatories (AIAPs)	**Acetaminophen**	17	Choi et al., 2008;Foster, 2007;Gómez et al., 2007; Guerra et al., 2014;Khan and Ongerth, 2005;Pereirea et al., 2015Radjenovic et al., 2007;Roberts and Thomas, 2006;Rosal et al., 2010;Snyder et al., 2006;Yu et al., 2006;Ashfaq et al 2017;Papageorgiou et al., 2016;Park et al., 2017;Shreve and Brennan, 2019; Thiebault et al., 2017; Wang et al., 2018;	15	Ashfaq et al 20 2009;Foster, 20 2014;Kasprzyk- Ongerth, 2005;I 2016;Park et al. 2019;Ternes, 19 2018;
	Codeine	7	Foster, 2007;Gómez et al., 2007; Guerra et al., 2014;Kasprzyk-Hordern et al., 2009;Rosal et al., 2010; Thiebault et al., 2017;Wick et al., 2009;	7	Yasojima et al., Guerra et al., 20 2009;Rosal et a al., 2009;
	Diclofenac	33	Ashfaq et al 2017;Bendz et al., 2005;Clara et al., 2005a;Clara et al., 2005b; Gómez et al., 2007;Jelic et al., 2012;Kasprzyk-Hordern et al., 2009;Kim et al., 2007;Lindqvist et al., 2005;Lishman et al., 2006;Martínez-Alcalá et al., 2017;Papageorgiou et al., 2016;Park et al., 2017;Paxéus, 2004;Pereirea et al., 2015Quintana et al., 2005;Radjenovic et al., 2009;Reif et al., 2008;Roberts and Thomas, 2006;Rosal et al., 2010;Santos et al., 2009; Santos et al., 2007;Shreve and Brennan, 2019;Snyder et al., 2006;Stumpf et al., 1999;Suárez et al., 2005;Tauxe-Wuersch et al., 2005;Thiebault et al., 2017;Thomas and Foster, 2005;Vieno et al., 2005;Weigel et al., 2004; Yu et al., 2006;Zorita et al., 2009;	35	Andreozzi et al 2005;Clara et a et al., 2009;Gór 2012;Kasprzyk- 2007;Kimura et 2005;Lishman e 2017;Muñoz et 2016;Park et al. 2005;Reif et al. 2006;Rosal et a al., 2007;Shreve 1999;Suárez et 2005;Ternes et 2017;Thomas a et al., 2006;Zor

	Ibuprofen	36	Ashfaq et al 2017;Bendz et al., 2005;Carballa et al., 2004;Clara et al., 2005a;Clara et al., 2005b; Gómez et al., 2007;Guerra et al., 2014;Kasprzyk-Hordern et al., 2009;Khan and Ongerth, 2005;Kimura et al 2007; Lindqvist et al., 2005;Lishman et al., 2006;Martínez-Alcalá et al., 2017;Nakada et al., 2006Paxéus, 2004;Pereirea et al., 2015Quintana et al., 2005;Radjenovic et al., 2009;Reif et al., 2008;Roberts and Thomas, 2006;Rodriguez et al., 2003;Rosal et al., 2010;Santos et al., 2009; Santos et al., 2007;Shreve and Brennan, 2019;Snyder et al., 2006;Stumpf et al., 1999;Suárez et al., 2005;Tauxe-Wuersch et al., 2005;Thiebault et al., 2017;Thomas and Foster, 2005;Vieno et al., 2005;Wang et al., 2018;Weigel et al., 2004; Yu et al., 2006;Zorita et al., 2009;	38	Andreozzi et al 2005;Carballa e al., 2005b; Coe 2007;Guerra et 2009;Khan and 2007;Kimura et et al., 2005;Lish al., 2017;Muño 2006Paxéus, 20 Thomas, 2006;I 2010;Santos et and Brennan, 2(1999;Suárez et 2005;Ternes et 2017;Thomas a 2005;Wang et a
	Indomethacin	9	Ashfaq et al 2017;Bendz et al., 2005;Lishman et al., 2006;Papageorgiou et al., 2016;Park et al., 2017;Radjenovic et al., 2009;Rosal et al., 2010;Stumpf et al., 1999;Wang et al., 2018;	10	Ashfaq et al 20 2006;Papageorg et al., 2010;Stur 2003;Ternes, 19
	Ketoprofen	24	Ashfaq et al 2017;Bendz et al., 2005;Jelic et al., 2012;Kasprzyk-Hordern et al., 2009;Khan and Ongerth, 2005;Kimura et al 2007; Lindqvist et al., 2005;Lishman et al., 2006;Martínez-Alcalá et al., 2017;Nakada et al., 2006Papageorgiou et al., 2016;Park et al., 2017;Quintana et al., 2005;Radjenovic et al., 2007;Rosal et al., 2010;Santos et al., 2009; Santos et al., 2007;Stumpf et al., 1999;Tauxe-Wuersch et al., 2005;Thiebault et al., 2017;Thomas and Foster, 2005;Vieno et al., 2005;Wang et al., 2018;Yu et al., 2006;	25	Andreozzi et al 2005;Jelic et al. 2009;Khan and 2005;Lindqvist 2006;Martínez- 2006Papageorg 2017;Quintana al., 2009; Santo 1999;Tauxe-Wu 1998;Thiebault 2005;Vieno et a 2006;

	Naproxen	31	Ashfaq et al 2017;Bendz et al., 2005;Carballa et al., 2004;Guerra et al., 2014;Jelic et al., 2012;Kasprzyk-Hordern et al., 2009;Khan and Ongerth, 2005;Kimura et al 2007; Lindqvist et al., 2005;Lishman et al., 2006;Martínez-Alcalá et al., 2017;Nakada et al., 2006Papageorgiou et al., 2016;Park et al., 2017;Paxéus, 2004;Quintana et al., 2005;Radjenovic et al., 2009;Reif et al., 2008;Rodriguez et al., 2003;Rosal et al., 2010;Santos et al., 2007;Shreve and Brennan, 2019;Snyder et al., 2006;Stumpf et al., 1999;Suárez et al., 2005;Thiebault et al., 2017;Thomas and Foster, 2005;Vieno et al., 2005;Wang et al., 2018;Yu et al., 2006;Zorita et al., 2009;	34	Andreozzi et al 2005;Carballa e al., 2012;Kaspr Ongerth, 2005; 2005;Lindqvist 2006;Martínez- 2006Papageorg 2017;Paxéus, 2 2008;Rodriguez et al., 2007;Shr 2006;Stumpf et al., 2003;Terne and Foster, 200
	Propyphenazone	1	Nakada et al., 2006	1	Nakada et al., 2
Antibiotics	**Clarithromycin**	6	Ghosh et al., 2009;Yasojima et al., 2006;Göbel et al., 2005; Guerra et al., 2014; Jelic et al., 2012;Park et al., 2017;	6	Göbel et al., 20 2017; Jelic et al al., 2006;
	Ofloxacin	11	Ashfaq et al 2017;Brown et al., 2006;Guerra et al., 2014;Jia et al., 2012;Lindberg et al., 2005, Peng et al., 2006;Radjenovic et al., 2009;Rosal et al., 2010;Wang et al., 2018;Wick et al., 2009;Zorita et al., 2009;	11	Andreozzi et al 2006;Guerra et al., 2005, Peng al., 2018;Wick
	Sulfadiazine	5	Ashfaq et al 2017; Gao et al., 2012a; Guerra et al., 2014;Papageorgiou et al., 2016;Peng et al., 2006;	5	Ashfaq et al 20 2014;Papageorg
	Sulfamethazine	5	Ashfaq et al 2017; Gao et al., 2012a; Guerra et al., 2014; Karthikeyan and Meyer, 2006;Sahar et al., 2011;	3	Ashfaq et al 20 2014

	Sulfamethoxazole	25	Bendz et al., 2005;Brown et al., 2006;Carballa et al., 2004;Choi et al., 2008;Clara et al., 2005b; Foster, 2007;Göbel et al., 2005; Gao et al., 2012a; Guerra et al., 2014Karthikeyan and Meyer, 2006;Kasprzyk-Hordern et al., 2009;Lindberg et al., 2005, Peng et al., 2006;Radjenovic et al., 2009;Reif et al., 2008;Rosal et al., 2010;Martin Ruel et al., 2010;Snyder et al., 2006;Watkinson et al., 2007;Wick et al., 2009;Ashfaq et al 2017;Gurke et al., 2015;Papageorgiou et al., 2016;Park et al., 2017;Shreve and Brennan, 2019;	26	Andreozzi et al 2005;Brown et al., 2008;Clara 2005; Gao et al al., 2015;Karthi Hordern et al., 2005, Muñoz et 2016;Park et al. 2008;Rosal et a and Brennan, 2 al., 2007;
	Trimethoprim	24	Batt 2006;Bendz et al., 2005;Brown et al., 2006;Choi et al., 2008;Foster, 2007;Ghosh et al., 2009; Guerra et al., 2014;Göbel et al., 2005;Gulkowska et al., 2008; Jelic et al., 2012;Karthikeyan and Meyer, 2006;Kasprzyk-Hordern et al., 2009;Lindberg et al., 2005, Paxéus, 2004;Radjenovic et al., 2009;Reif et al., 2008;Roberts and Thomas, 2006;Rosal et al., 2010;Snyder et al., 2006;Watkinson et al., 2007;Gurke et al., 2015;Papageorgiou et al., 2016;Park et al., 2017;Shreve and Brennan, 2019;	26	Andreozzi et al 2005;Brown et 2007; Guerra et 2005;Gulkowsk et al., 2012;Kar Hordern et al., 2005, Lindberg 2016;Park et al. 2008;Roberts a 2010;Shreve an 2006;Ternes et
Beta-Blockers	**Atenolol**	12	Alder et al., 2010;Bendz et al., 2005;Kasprzyk-Hordern et al., 2009;Maurer et al., 2007;Radjenovic et al., 2009;Rosal et al., 2010;Wick et al., 2009;Gurke et al., 2015; Jelic et al., 2012;Papageorgiou et al., 2016;Park et al., 2017; Thiebault et al., 2017;	14	Alder et al., 201 2015; Jelic et al 2009;Maurer et 2009;Papageorg 2017;Paxéus, 2 2003; Thiebault
	Metoprolol	14	Alder et al., 2010;Ashfaq et al 2017;Gurke et al., 2015;Kasprzyk-Hordern et al., 2009;Maurer et al., 2007;Papageorgiou et al., 2016;Park et al., 2017;Paxéus, 2004;Radjenovic et al., 2009;Rosal et al., 2010;Shreve and Brennan, 2019;Thiebault et al., 2017;Wang et al., 2018;Wick et al., 2009;	16	Alder et al., 201 2017;Gurke et a 2009;Maurer et 2016;Park et al. 2010;Shreve an 2003;Ternes, 19 2018;Wick et a

	Propranolol	13	Alder et al., 2010;Bendz et al., 2005;Kasprzyk-Hordern et al., 2009;Maurer et al., 2007;Radjenovic et al., 2009;Roberts and Thomas, 2006;Rosal et al., 2010;Wick et al., 2009;Ashfaq et al 2017;Gurke et al., 2015;Papageorgiou et al., 2016;Park et al., 2017; Wang et al., 2018;	16	Alder et al., 201 2017;Bendz et a al., 2015;Gurke 2009;Maurer et 2016;Park et al. 2006;Rosal et a 1998;Wick et a
	Sotalol	7	Alder et al., 2010; Ashfaq et al 2017;Maurer et al., 2007; Jelic et al., 2012;Radjenovic et al., 2009; Wang et al., 2018;Wick et al., 2009;	7	Alder et al., 201 2007; Jelic et al 2018;Wick et a
Diuretics	**Furosemide**	4	Kasprzyk-Hordern et al., 2009;Rosal et al., 2010;Papageorgiou et al., 2016;Park et al., 2017;	4	Kasprzyk-Hord 2016;Park et al.
	Hydrochlorothiazide	2	Radjenovic et al., 2009; Jelic et al., 2012;Rosal et al., 2010;	1	Jelic et al., 201
Lipid-regulators	**Bezafibrate**	14	Clara et al., 2005a;Clara et al., 2005b; Kasprzyk-Hordern et al., 2009;Lindqvist et al., 2005; Jelic et al., 2012;Pereirea et al., 2015Quintana et al., 2005;Radjenovic et al., 2009;Rosal et al., 2010;Stumpf et al., 1999;Vieno et al., 2005;Gurke et al., 2015;Papageorgiou et al., 2016;Park et al., 2017;	13	Andreozzi et al 2005b; Gurke e 2009;Lindqvist 2012;Papageorg 2017;Quintana al., 1999;Terne
	Fenofibrate	1	Papageorgiou et al., 2016;	3	Andreozzi et al 1998;
	Gemfibrozil	16	Ashfaq et al 2017;Bendz et al., 2005;Jelic et al., 2012;Khan and Ongerth, 2005;Lishman et al., 2006;Papageorgiou et al., 2016;Paxéus, 2004;Pereirea et al., 2015Radjenovic et al., 2009;Rosal et al., 2010;Shreve and Brennan, 2019;Snyder et al., 2006;Stumpf et al., 1999;Thiebault et al., 2017;Wang et al., 2018;Yu et al., 2006;	16	Andreozzi et al 2005;Jelic et al. al., 2007;Lishm 2009;Papageorg al., 2010;Shreve 1999;Ternes, 19 2018;

Psychiatric Medications	**Carbamazepine**	29	Ashfaq et al 2017;Bendz et al., 2005;Choi et al., 2008;Clara et al., 2005a;Clara et al., 2005b; Conti et al., 2011;Foster, 2007;Gao et al., 2012b;Gómez et al., 2007;Gurke et al., 2015;Jelic et al., 2012;Kasprzyk-Hordern et al., 2009;Khan and Ongerth, 2005;Martínez-Alcalá et al., 2017;Nakada et al., 2006Papageorgiou et al., 2016;Park et al., 2017;Paxéus, 2004;Radjenovic et al., 2009;Reif et al., 2008;Rosal et al., 2010;Santos et al., 2009; Santos et al., 2007;Shreve and Brennan, 2019;Snyder et al., 2006;Suárez et al., 2005;Thiebault et al., 2017;Wang et al., 2018;Wick et al., 2009;	32	Andreozzi et al 2005;Choi et al 2005b; Coetsier 2012b;Gómez e al., 2012;Kaspr Ongerth, 2005; 2017;Muñoz et 2006Papageorg 2017;Paxéus, 2 2010;Santos et and Brennan, 2 2003;Ternes, 19 2018;Wick et a
	Diazepam	6	Ashfaq et al 2017;Jelic et al., 2012;Reif et al., 2008;Suárez et al., 2005;Thiebault et al., 2017;Wang et al., 2018;	9	Ashfaq et al 20 2012;Reif et al. 1998;Thiebault al., 2009;
	Lorazepam	2	Pereirea et al., 2015; Jelic et al., 2012;	2	Coetsier et al., 2
	Paroxetine	2	Metcalfe et al. 2010;	1	Metcalfe et al. 2

Table I-S2: Numbers of papers and references examined to find the raw sewage sludge concentration and digested sluc
PhACs in the anaerobic digestion (AD) process of the sewage sludge.

Groups	Pharmaceutical compound	Numbers of papers	References for raw sewage sludge	Numbers of papers	Ref
Analgesics/Anti-inflammatories (AIAPs)	**Acetaminophen**	8	Gao et al., 2012b;Gao et al., 2012b;Khan and Ongerh 2002;Khan and Ongerh 2002;Mailler et al., 2016;Martín et al., 2015;Martín et al., 2015;Narumiya et al., 2013;Phan et al., 2018;Radjonevic et al., 2009;Radjonevic et al., 2009;Yang et al., 2016;	6	Mailler et a et al., 2018; 2014; Yang
	Codeine	1	Ivanova et al., 2018;	1	Ivanova et a
	Diclofenac	13	Gonzalez-Gil et al., 2016;Gonzalez-Gil et al., 2018;Ivanova et al., 2018;Jones et al., 2014;Martín et al., 2012a;Martín et al., 2012b;Martín et al., 2015;Narumiya et al., 2013;Phan et al., 2018;Radjonevic et al., 2009;Stasinakis et al., 2013;Ternes et al., 2004;Yang et al., 2016;	9	Yang et al., 2011;Martí 2015;Narun 2009;Phan
	Ibuprofen	11	Gonzalez-Gil et al., 2016;Gonzalez-Gil et al., 2018;Jones et al., 2014;Khan and Ongerh 2002;Martín et al., 2012a;Martín et al., 2012b;Martín et al., 2015;Phan et al., 2018;Radjonevic et al., 2009;Ternes et al., 2004;Yang et al., 2016;	6	Gonzalez-G 2012b;Mart 2018;Radjo
	Indomethacin	0			
	Ketoprofen	2	Martín et al., 2012b;Radjonevic et al., 2009;	3	Martín et al 2012b;Radj

	Naproxen	7	Gonzalez-Gil et al., 2016;Gonzalez-Gil et al., 2018;Khan and Ongerh 2002;Martín et al., 2012b;Martín et al., 2015;Phan et al., 2018;Yang et al., 2016;	7	Khan and C 2015;Martín 2015;Phan 2013;Yang
	Propyphenazone	0		0	
Antibiotics	**Clarithromycin**	2	Ivanova et al., 2018;Narumiya et al., 2013;	2	Okuda et al
	Ofloxacin	7	Mailler et al., 2016;Martín et al., 2015;Radjonevic et al., 2009;Gao et al., 2012b;Jia et al., 2012;Jones et al., 2014;Narumiya et al., 2013;	3	Radjonevic 2009;Yan e
	Sulfadiazine	2	Gao et al., 2012b;Yan et al., 2014	1	Gao et al., 2
	Sulfamethazine	2	Gao et al., 2012b;Yan et al., 2014	1	Gonzalez-G
	Sulfamethoxazole	10	Mailler et al., 2016;Martín et al., 2012b;Martín et al., 2015; Narumiya et al., 2013;Phan et al., 2018;Radjonevic et al., 2009;Yan et al., 2014Mailler et al., 2016;Phan et al., 2018;	4	Gao et al., 2 Gil et al., 20
	Trimethoprim	8	Gonzalez-Gil et al., 2016;Gonzalez-Gil et al., 2018;Martín et al., 2012b;Martín et al., 2015;Phan et al., 2018;Radjonevic et al., 2009;Yan et al., 2014Yang et al., 2016;	6	Gonzalez-G 2011;Martín 2018;Radjo
Beta-Blockers	**Atenolol**	6	Yang et al., 2016;Martín et al., 2015;Martín et al., 2015;Radjonevic et al., 2009;Radjonevic et al., 2009;	6	Radjonevic 2011;Malm and Magnér al., 2018;
	Metoprolol	3	Ivanova et al., 2018;Ivanova et al., 2018;Narumiya et al., 2013;	2	Scheurer et
	Propranolol	6	Jones et al., 2014;Mailler et al., 2016;Martín et al., 2012b;Martín et al., 2015;Radjonevic et al., 2009;Scheurer et al., 2010;	4	Mailler et a 2015;Radjo 2010;
	Sotalol	1	Scheurer et al., 2010;	2	Scheurer et

Diuretics	**Furosemide**	0		2	Jelic et al.,
	Hydrochlorothiazide	1	Radjonevic et al., 2009;	3	Jelic et al., 2015;Radjo
Lipid-regulators	**Bezafibrate**	1	Martín et al., 2015;	1	Jelic et al.,
	Fenofibrate	1	Ivanova et al., 2018;	2	Jelic et al.,
	Gemfibrozil	7	Khan and Ongerh 2002;Martín et al., 2012b;Martín et al., 2015;Phan et al., 2018;Radjonevic et al., 2009;Yan et al., 2014Yang et al., 2016;	4	Gonzalez-G Magnér, 20 2016;
Psychiatric Medications	**Carbamazepine**	14	Gao et al., 2012b;Gonzalez-Gil et al., 2016;Gonzalez-Gil et al., 2018;Ivanova et al., 2018;Khan and Ongerh 2002;Mailler et al., 2016;Martín et al., 2012b;Martín et al., 2015;Narumiya et al., 2013;Phan et al., 2018;Radjonevic et al., 2009;Ternes et al., 2004;Yan et al., 2014Yang et al., 2016;	8	Ivanova et a 2016;Malm 2015;Narun 2018;Radjo
	Diazepam	2	Radjonevic et al., 2009;Ternes et al., 2004;	2	Gonzalez-G Magnér, 20
	Lorazepam	1	Malmborg and Magnér, 2015;	2	Gonzalez-G Magnér, 20
	Paroxetine	1	Radjonevic et al., 2009;	3	Gonzalez-G Magnér, 20

Table I-S3: Numbers of papers and references examined to find the mass load influent and secondary effluent (mg com
PhACs in the WWTPs studied by these papers.

Groups	Pharmaceutical compound	Numbers of papers	References for mass load influent (mg compound/1000 inh.d)	Numbers of papers	Referenc
Analgesics/Anti-inflammatories (AIAPs)	**Acetaminophen**	7	Gracia-Lor et al., 2012;Papageorgiou et al., 2016;Pereira et al., 2015;Radjenovic et al., 2009;Subedi et al., 2017;Thiebault et al., 2017;Verlicchi et al., 2012;Yan et al., 2014;Yu et al., 2013;	9	Gracia-Lo 2016;Pere 2009;Sube 2017;Verl et al., 201
	Codeine	3	Gurke et al., 2015;Subedi et al., 2017;Thiebault et al., 2017;	3	Gurke et a et al., 201
	Diclofenac	9	Gracia-Lor et al., 2012;Jelic et al., 2012;Papageorgiou et al., 2016;Pereira et al., 2015;Samaras et al., 2013;Thiebault et al., 2017;Yan et al., 2014;Yu et al., 2013;Zorita et al., 2009;	10	Gracia-Lo 2016;Pere 2009;Sam 2017;Verl et al., 201
	Ibuprofen	9	Castiglioni et al., 2005;Gracia-Lor et al., 2012;Pereira et al., 2015;Samaras et al., 2013;Subedi et al., 2017;Thiebault et al., 2017;Yan et al., 2014;Yu et al., 2013;Zorita et al., 2009;	11	Castiglion 2012;Pere 2009;Sam 2017;Thie 2012;Yan al., 2013;
	Indomethacin	1	Papageorgiou et al., 2016;	2	Papageorg
	Ketoprofen	2	Papageorgiou et al., 2016;Thiebault et al., 2017;	3	Papageorg 2017;Verl
	Naproxen	3	Papageorgiou et al., 2016;Thiebault et al., 2017;Zorita et al., 2009;	4	Papageorg 2017;Verl
	Propyphenazone	1	Papageorgiou et al., 2016;	1	Verlicchi

Antibiotics	**Clarithromycin**	1	Papageorgiou et al., 2016;	2	Pereira et
	Ofloxacin	9	Castiglioni et al., 2005;Golet et al., 2002;Gracia-Lor et al., 2012;Leung et al., 2012;Li and Zhang, 2011;Lindberg et al., 2005;Peng et al., 2006;Yan et al., 2014;Zorita et al., 2009;	11	Castiglion 2002;Grac 2012;Li ar 2005;Peng 2009;Verl 2014;Zori
	Sulfadiazine	4	Li and Zhang, 2011;Papageorgiou et al., 2016;Peng et al., 2006;Yan et al., 2014;	4	Li and Zha 2016;Peng
	Sulfamethazine	8	Castiglioni et al., 2005;Gracia-Lor et al., 2012;Karthikeyan and Meyer, 2006;Leung et al., 2012;Li and Zhang, 2011;Lindberg et al., 2005;Peng et al., 2006;Yan et al., 2014;	12	Castiglion 2012;Kart al., 2012;L 2005;Peng 2009;Yan 2016;Subo
	Sulfamethoxazole	2	Papageorgiou et al., 2016;Subedi et al., 2017;	2	Papageorg
	Trimethoprim	10	Gracia-Lor et al., 2012;Gulkowska et al., 2008;Karthikeyan and Meyer, 2006;Leung et al., 2012;Li and Zhang, 2011;Lindberg et al., 2005;Papageorgiou et al., 2016;Subedi et al., 2017;Watkinson et al., 2007;Yan et al., 2014;	13	Gracia-Lo 2008;Jelic 2006;Leur 2011;Lind 2016;Radj 2017;Verl 2007;Yan
Beta-Blockers	**Atenolol**	6	Collado et al., 2014;Gurke et al., 2015;Papageorgiou et al., 2016;Subedi et al., 2017;Thiebault et al., 2017;Wick et al., 2009;	7	Collado et 2015;Papa 2017;Thie 2012;Wicl
	Metoprolol	3	Papageorgiou et al., 2016;Thiebault et al., 2017;Wick et al., 2009;	4	Papageorg 2015;Thie
	Propranolol	2	Papageorgiou et al., 2016;Subedi et al., 2017;	4	Papageorg 2015;Subo
	Sotalol	1	Papageorgiou et al., 2016;	2	Pereira et

Diuretics	**Furosemide**	1	Papageorgiou et al., 2016;	1	Verlicchi
	Hydrochlorothiazide	1	Papageorgiou et al., 2016;	3	Papageorg 2015;Verl
Lipid-regulators	**Bezafibrate**	6	Gracia-Lor et al., 2012;Papageorgiou et al., 2016;Pereira et al., 2015;Radjenovic et al., 2009;Yan et al., 2014;Yu et al., 2013;	7	Gracia-Lo 2012;Papa 2015;Verl et al., 201
	Fenofibrate	2	Papageorgiou et al., 2016;Pereira et al., 2015;	2	Papageorg
	Gemfibrozil	2	Papageorgiou et al., 2016;Thiebault et al., 2017;	3	Papageorg 2017;Verl
Psychiatric Medications	**Carbamazepine**	7	Castiglioni et al., 2005;Gurke et al., 2015;Papageorgiou et al., 2016;Subedi et al., 2017;Thiebault et al., 2017;Wick et al., 2009;Yan et al., 2014;	10	Castiglion et al., 201 2016;Radj 2017;Thie 2012;Wicl
	Diazepam	2	Subedi et al., 2017;Thiebault et al., 2017;	3	Subedi et 2017;Verl
	Lorazepam	0		0	
	Paroxetine	1	Pereira et al., 2015;	1	Pereira et

Table I-S4: Numbers of papers and references examined to find the removal efficiency (RE) in water line for conventio the selected PhACs. The RE values for the references underline are evaluated by means of eq. I.1.

Groups	Pharmaceutical compound	Numbers of papers	References for RE in water line for CA
Analgesics/ Anti-inflammato	**Acetaminophen**	20	Ashfaq et al., 2017;Choi et al., 2008;De la Torre et al., 2015; Foster, 2007;Gh et al., 2007; Kasprzyk-Hordern et al., 2009;Khan and Ongerth, 2005;Majewsk al., 2014;Radjenovic et al., 2007;Radjenovic et al., 2009;Roberts and Thomas 2017; Wang et al., 2018; Yan et al., 2014;Yu et al., 2006

	Codeine	8	Foster, 2007;Ghosh et al., 2009; Guerra et al., 2014;Karthikeyan and Meyer, 2 2017;Wick et al, 2009; Yadav et al., 2019;
	Diclofenac	35	Ashfaq et al., 2017;Bendz et al., 2005;Bernhard et al., 2006;Clara et al., 2004; la Torre et al., 2015;Gómez et al., 2007; Jelic et al., 2011; Jelic et al., 2012; Jo 2009;Kimura et al., 2007; Kreuzinger et al., 2004;Lindqvist et al., 2005;Lishm 2017; Majewsky et al., 2011;Park et al., 2017;Paxéus, 2004;Radjenovic et al., al., 2010;Santos et al., 2007; Santos et al., 2009; Stasinakis et al., 2013; Stum 2005;Tadkaew et al., 2010;Ternes, 1998; Thiebault et al., 2017; Urase et al., 2 2010;Zorita et al., 2009
	Ibuprofen	45	Ashfaq et al., 2017;Bendz et al., 2005;Bernhard et al., 2006;Carballa et al., 20 2006;Clara et al., 2004;Clara et al., 2005;De la Torre et al., 2015Fernandez-Fo 2009;Gros et al., 2010; Guerra et al., 2014; Jones et al., 2007; Joss et al., 2005 Ongerth, 2005;Kimura et al., 2007;Lindberg et al., 2005;Lishman et al., 2006; al., 2017; Paxéus, 2004; Petrie et al., 2014; Radjenovic et al., 2007;Roberts an 2003;Rosal et al., 2010;Santos et al., 2009; Santos et al., 2007;Stumpf et al., 2005;Tauxe-Wuersch et al., 2005;Ternes, 1998; Thiebault et al., 2017; Urase al., 2018; Yan et al., 2014;Yu et al., 2006;Zorita et al., 2009
	Indomethacin	9	Ashfaq et al., 2017;Lishman et al., 2006; Jelic et al., 2011; Park et al., 2017;R 2010;Stumpf et al., 1999;Ternes, 1998; Wang et al., 2018;
	Ketoprofen	25	Ashfaq et al., 2017;Bendz et al., 2005;De la Torre et al., 2015; Jelic et al., 201 Meyer, 2006;Kasprzyk-Hordern et al., 2009;Khan and Ongerth, 2005;Kimura 2005;Lishman et al., 2006;Nakada et al., 2006; Martínez-Alcalá et al., 2017; P 2007;Radjenovic et al., 2009;Santos et al., 2007; Santos et al., 2009; Stasinal 1999;Tadkaew et al., 2010; Thiebault et al., 2017;Urase et al., 2005;Vieno et a
	Naproxen	36	Ashfaq et al., 2017;Bendz et al., 2005;Carballa et al., 2004;Carballa et al., 200 Fontaina et al., 2012;Gros et al., 2010; Guerra et al., 2014; Jelic et al., 2011; J 2005;Kasprzyk-Hordern et al., 2009;Khan and Ongerth, 2005;Kimura et al., 2 2006;Nakada et al., 2006; Martínez-Alcalá et al., 2017; Park et al., 2017;Paxé et al., 2007;Radjenovic et al., 2009;Rodriguez et al., 2003;Rosal et al., 2010;S Stasinakis et al., 2013;Stumpf et al., 1999;Suárez et al., 2005;Tadkaew et al., 2005;Vieno et al., 2005;Zorita et al., 2009; Wang et al., 2018
	Propyphenazone	5	Ashfaq et al., 2017;Nakada et al., 2006;Radjenovic et al., 2007; Radjenovic et

Antibiotics	**Clarithromycin**	8	Castiglioni et al., 2006;Ghosh et al., 2009;Göbel et al., 2005;Göbel et al., 200 Park et al., 2017;Sahar et al., 2011
	Ofloxacin	17	Ashfaq et al., 2017;Brown et al., 2006;Castiglioni et al., 2006;Li and Zhang, 2 2014; Jia et al., 2012 Peng et al., 2006; Petrie et al., 2014; Radjenovic et al., 2 2005; Vieno et al., 2007;Wang et al., 2018; Xu et al., 2007; Yan et al., 2014;
	Sulfadiazine	7	Ashfaq et al., 2017;García-Galán et al., 2011; Gao et al., 2012a; Li and Zhang Yan et al., 2014
	Sulfamethazine	3	Li and Zhang, 2011; Jelic et al., 2011; Yan et al., 2014
	Sulfamethoxazole	32	Ashfaq et al., 2017;Brown et al., 2006;Carballa et al., 2004;Carballa et al., 20 2008;Clara et al., 2005b;De la Torre et al., 2015; Foster, 2007;García-Galán e al., 2005;Göbel et al., 2007; Gao et al., 2012a;Gros et al., 2010;Gurke et al., 2 Meyer, 2006;Kasprzyk-Hordern et al., 2009;Kreuzinger et al., 2004;Li and Zh 2005;Majewsky et al., 2011;Park et al., 2017;Peng et al., 2006;Radjenovic et a al., 2010;Sahar et al., 2011;Xia et al., 2012;Xu et al., 2007; Yan et al., 2014
	Trimethoprim	22	Batt 2006;Bendz et al., 2005;Brown et al., 2006;Choi et al., 2008;Fernandez-F et al., 2009;Göbel et al., 2007;Gulkowska et al., 2008; Jelic et al., 2011; Jelic 2006;Kasprzyk-Hordern et al., 2009;Li and Zhang, 2011;Lindberg et al., 2005 2004;Radjenovic et al., 2009;Rosal et al., 2010;Sahar et al., 2011;Xue et al., 2
Beta-Blockers	**Atenolol**	16	Alder et al., 2010;Carucci et al., 2006;Castiglioni et al., 2006;Gros et al., 2010 Jelic et al., 2012; Karthikeyan and Meyer, 2006;Maurer et al., 2007;Park et al al., 2010; Thiebault et al., 2017; Vieno et al., 2005; Vieno et al., 2007;Wick et
	Metoprolol	16	Alder et al., 2010;Ashfaq et al., 2017; Jelic et al., 2011; Karthikeyan and Mey 2017;Paxéus, 2004; Radjenovic et al., 2009;Rosal et al., 2010;Ternes, 1998; T Vieno et al., 2007; Wang et al., 2018; Wick et al, 2009; Yan et al., 2014
	Propranolol	12	Alder et al., 2010;Gurke et al., 2015;Karthikeyan and Meyer, 2006;Maurer et 2014; Rosal et al., 2010; Radjenovic et al., 2009;Ternes, 1998; Wang et al., 20
	Sotalol	9	Alder et al., 2010;Gurke et al., 2015; Jelic et al., 2011; Jelic et al., 2012; Mau et al., 2007;Wick et al, 2009; Wang et al., 2018
Diuretics	**Furosemide**	7	Castiglioni et al., 2006;Gros et al., 2010;Gros et al., 2010; Jelic et al., 2011; Je 2006; Park et al., 2017; Rosal et al., 2010;
	Hydrochlorothiazide	4	Castiglioni et al., 2006; Jelic et al., 2011; Jelic et al., 2012; Radjenovic et al.,

Lipid-regulators	**Bezafibrate**	19	Castiglioni et al., 2006;Clara et al., 2004;Clara et al., 2005a;Clara et al., 2005b Jelic et al., 2011; Jelic et al., 2012; Kasprzyk-Hordern et al., 2009;Kreuzinger al., 2017; Petrie et al., 2014; Radjenovic et al., 2007;Radjenovic et al., 2009;S al., 2005; Yan et al., 2014
	Fenofibrate	3	Ternes, 1998; Jelic et al., 2011
	Gemfibrozil	14	Ashfaq et al., 2017;Bendz et al., 2005; Jelic et al., 2011; Jelic et al., 2012; Kha 2006;Paxéus, 2004;Radjenovic et al., 2007;Rosal et al., 2010;Ternes, 1998; Th Wang et al., 2018; Yu et al., 2006
Psychiatric Medications	**Carbamazepine**	39	Ashfaq et al., 2017;Bendz et al., 2005;Bernhard et al., 2006;Castiglioni et al., 2004;Clara et al., 2005a;Clara et al., 2005b;De la Torre et al., 2015; Foster, 20 2007;Gurke et al., 2015; Jelic et al., 2011; Jelic et al., 2012; Joss et al., 2005;K Ongerth, 2005;Kreuzinger et al., 2004; Martínez-Alcalá et al., 2017;Nakada et Petrie et al., 2014; Radjenovic et al., 2007;Radjenovic et al., 2009;Rosal et al., 2009; Suárez et al., 2005;Tadkaew et al., 2010; Thiebault et al., 2017; Urase et al., 2018; Wick et al, 2009;Xue et al., 2010; Yan et al., 2014; Zorita et al., 200
	Diazepam	7	Ashfaq et al., 2017; Jelic et al., 2011; Kreuzinger et al., 2004; Petrie et al., 201 2017; Wang et al., 2018
	Lorazepam	2	Jelic et al., 2011; Jelic et al., 2012
	Paroxetine	1	Radjenovic et al., 2007

Table I-S5: Numbers of papers and references examined to find the removal efficiency (RE) in water line for process of the selected PhACs. The RE values for the references underline are evaluated by means of eq. I.1.

Groups	Pharmaceutical compound	Numbers of papers	References for RE in water line for MBR
Analgesics /Anti	**Acetaminophen**	8	De la Torre et al., 2015Maeng et al., 2013; Park et al., 2017; Radjenc al., 2009;Snyder et al., 2006;Tadkaew et al., 2010; Tambosi et al., 20

	Codeine	1	Yadav et al., 2019;
	Diclofenac	22	Bernhard et al., 2006;Clara et al., 2004;Clara et al., 2005a;Clara et al De la Torre et al., 2015;Joss et al., 2005;Kimura et al., 2007;Kreuzing 2015;Maeng et al., 2013;Park et al., 2017;Quintana et al., 2005;Radj 2007;Radjenovic et al., 2009;Reif et al., 2008;Roberts and Thomas, 2 2006;Tauxe-Wuersch et al., 2005;Urase et al., 2005;Weigel et al., 20
	Ibuprofen	18	Bernhard et al., 2006;Clara et al., 2004;Clara et al., 2005b;De la Torr 2005;Kimura et al., 2007;Kreuzinger et al., 2004; Luo et al., 2015;Ma al., 2005;Radjenovic et al., 2007;Radjenovic et al., 2009;Reif et al., 2 2006;Tauxe-Wuersch et al., 2005;Urase et al., 2005;Weigel et al., 20
	Indomethacin	3	Radjenovic et al., 2007; Radjenovic et al., 2009; Rosal et al., 2010;
	Ketoprofen	14	De la Torre et al., 2015; Luo et al., 2015;Kimura et al., 2007;Park et a 2005;Radjenovic et al., 2007;Radjenovic et al., 2009;Rosal et al., 201 2010;Tauxe-Wuersch et al., 2005; Tambosi et al., 2010;Urase et al., al., 2006
	Naproxen	15	De la Torre et al., 2015Joss et al., 2005;Kimura et al., 2007;Maeng e 2017;Quintana et al., 2005; Luo et al., 2015;Radjenovic et al., 2007; al., 2008;Santos et al., 2009; Snyder et al., 2006;Tadkaew et al., 201 et al., 2005
	Propyphenazone	1	Radjenovic et al., 2009;
Antibiotics	**Clarithromycin**	3	Göbel et al., 2007;Sahar et al., 2011;Yasojima et al., 2006
	Ofloxacin	2	Radjenovic et al., 2009;Rosal et al., 2010
	Sulfadiazine	1	Xia *et al.*, 2012;
	Sulfamethazine	1	García-Galán et al., 2011;
	Sulfamethoxazole	14	De la Torre et al., 2015;Joss et al., 2005;Kreuzinger et al., 2004;Park 2007;Radjenovic et al., 2009;Reif et al., 2008;Sahar et al., 2011;Snyd al., 2010; Tambosi et al., 2010;Watkinson et al., 2007;Xia et al., 201

	Trimethoprim	9	Göbel et al., 2007;Park et al., 2017;Radjenovic et al., 2009;Roberts a al., 2011;Snyder et al., 2006;Tadkaew et al., 2010; Tambosi et al., 20
Beta-Blockers	**Atenolol**	3	Park et al., 2017;Radjenovic et al., 2007;
	Metoprolol	4	De la Torre et al., 2015; Radjenovic et al., 2007; Radjenovic et al., 2
	Propranolol	1	Radjenovic et al., 2009
	Sotalol	2	Alder et al., 2010; Radjenovic et al., 2009
Diuretics	**Furosemide**	1	Park et al., 2017;
	Hydrochlorothiazide	3	Radjenovic et al., 2007;Radjenovic et al., 2009;Rosal et al., 2010
Lipid-regulators	**Bezafibrate**	10	Clara et al., 2004;Clara et al., 2005a;Clara et al., 2005b;Kreuzinger e 2013;Park et al., 2017;Quintana et al., 2005;Radjenovic et al., 2007; et al., 2010
	Fenofibrate	0	
	Gemfibrozil	7	Maeng et al., 2013; Luo et al., 2015;Radjenovic et al., 2007;Radjeno 2006;Stumpf et al., 1999;Urase et al., 2005
Psychiatric Medications	**Carbamazepine**	17	Bernhard et al., 2006;Clara et al., 2004;Clara et al., 2005a;Clara et al 2015Joss et al., 2005;Kreuzinger et al., 2004; Luo et al., 2015;Maeng 2017;Radjenovic et al., 2009;Reif et al., 2008;Snyder et al., 2006;Ter 2005;Vieno et al., 2005;Zorita et al., 2009
	Diazepam	1	Reif et al., 2008;
	Lorazepam	0	
	Paroxetine	0	

Table I-S6: Numbers of papers and references examined to find the removal efficiency (RE) in water line f
sludge (IFAS) systems or moving bed biofilm reactor (MBBR) process of the selected PhACs.

Groups	Pharmaceutical compound	Numbers of papers	References for RE in water line for IFAS or M
Analgesics/Anti-inflammatories (AIAPs)	**Acetaminophen**	4	Ba et al., 2014;Luo et al., 2014;Luo et al., 2015;Shreve and Brenn
	Codeine	1	Yadav et al., 2019;
	Diclofenac	9	Alvarino et al., 2016;De la Torre et al., 2015Escola Casas and Bes Jewell et al., 2016; Luo et al., 2014;Luo et al., 2015;Ooi et al., 201
	Ibuprofen	7	Alvarino et al., 2016;Casas et al., 2015;De la Torre et al., 2015; Fa 2014; Luo et al., 2015;Ooi et al., 2018
	Indomethacin	0	
	Ketoprofen	4	De la Torre et al., 2015; Falas et al., 2012;Luo et al., 2014; Luo et
	Naproxen	5	De la Torre et al., 2015; Falas et al., 2012;Luo et al., 2014; Luo et Brennan, 2019;
	Propyphenazone	0	
Antibiotics	**Clarithromycin**	2	Falas et al., 2013;Ooi et al., 2018;
	Ofloxacin	0	
	Sulfadiazine	2	Escola Casas et al., 2015;Ooi et al., 2018;
	Sulfamethazine	0	
	Sulfamethoxazole	6	Alvarino et al., 2016;Casas et al., 2015;Escola Casas and Bester, 2 and Brennan, 2019;
	Trimethoprim	6	Alvarino et al., 2016;Casas et al., 2015; De la Torre et al., 2015; E 2015; Falas et al., 2013;Ooi et al., 2018;Shreve and Brennan, 201
Beta-Blockers	**Atenolol**	4	Casas et al., 2015;Escola Casas et al., 2015;Falas et al., 2012;Ooi
	Metoprolol	4	Casas et al., 2015; Escola Casas et al., 2015; Ooi et al., 2018;Shre

	Propranolol	4	Casas et al., 2015; De la Torre et al., 2015; Escola Casas et al., 20
	Sotalol	0	
Diuretics	**Furosemide**	0	
	Hydrochlorothiazide	0	
Lipid-regulators	**Bezafibrate**	0	
	Fenofibrate	0	
	Gemfibrozil	4	Falas et al., 2012;Luo et al., 2014;Luo et al., 2015;Shreve and Bre
Psychiatric Medications	**Carbamazepine**	9	Alvarino et al., 2016;Casas et al., 2015;De la Torre et al., 2015Esc et al., 2013;Luo et al., 2014;Luo et al., 2015;Ooi et al., 2018;Shrev
	Diazepam	1	Alvarino *et al*., 2016;
	Lorazepam	0	
	Paroxetine	0	

Table I-S7: Numbers of papers and references examined to find the removal efficiency (RE) in sludge line for the anae selected PhACs.

Groups	**Pharmaceutical compound**	**Numbers of papers**	**References for RE in the AD process**
Analgesics/Anti-inflammatories (AIAPs)	**Acetaminophen**	6	Gonzalez-Gil et al., 2019;Narumiya et al., 2013;Phan et al., 2018;Radje 2016;Yang et al., 2017;
	Codeine	1	Gonzalez-Gil et al., 2019;
	Diclofenac	13	Gonzalez-Gil et al., 2019;Jelic et al., 2012;Narumiya et al., 2013;Phan 2009;Samaras et al. 2014;Yang et al., 2016;Yang et al., 2017;Carballa e 2007a;Carballa et al., 2007b;Zhou et al., 2017;Malmborg and Magner,

	Ibuprofen	12	Gonzalez-Gil et al., 2016;Martín et al., 2012;Phan et al., 2018;Radjenov 2013;Samaras et al. 2014;Yang et al., 2016;Yang et al., 2017;Carballa e 2007a;Carballa et al., 2007b;Malmborg and Magner, 2015
	Indomethacin	1	Narumiya et al., 2013
	Ketoprofen	3	Narumiya et al., 2013;Radjenovic et al., 2009; Samaras et al. 2014
	Naproxen	9	Gonzalez-Gil et al., 2016;Martín et al., 2012;Phan et al., 2018;Samaras et al., 2016;Carballa et al., 2006;Carballa et al., 2007a;Malmborg and M
	Propyphenazone	0	
antibiotics	**Clarithromycin**	3	Gonzalez-Gil et al., 2019;Jelic et al., 2012;Narumiya et al., 2013;
	Ofloxacin	2	Narumiya et al., 2013;Radjenovic et al., 2009;
	Sulfadiazine	0	
	Sulfamethazine	0	
	Sulfamethoxazole	8	Gonzalez-Gil et al., 2016;Gonzalez-Gil et al., 2019;Narumiya et al., 20 al., 2009;Yang et al., 2017;Carballa et al., 2006;Carballa et al., 2007b;
	Trimethoprim	8	Gonzalez-Gil et al., 2016;Gonzalez-Gil et al., 2019;Narumiya et al., 20 al., 2009;Yang et al., 2016;Yang et al., 2017;Malmborg and Magner, 2
Beta-Blockers	**Atenolol**	6	Gonzalez-Gil et al., 2019;Narumiya et al., 2013;Phan et al., 2018;Radje 2016;Yang et al., 2017
	Metoprolol	1	Gonzalez-Gil et al., 2019;
	Propranolol	3	Martín et al., 2012;Radjenovic et al., 2009;Narumiya et al., 2013;
	Sotalol	0	
Diuretics	**Furosemide**	1	Jelic et al., 2012;
	Hydrochlorothiazide	2	Jelic et al., 2012;Radjenovic et al., 2009;
Lipid-reg	**Bezafibrate**	2	Gonzalez-Gil et al., 2019;Narumiya et al., 2013;

	Fenofibrate	0	
	Gemfibrozil	4	Phan et al., 2018;Radjenovic et al., 2009;Yang et al., 2016;Yang et al.,
Psychiatric Medications	**Carbamazepine**	12	Gonzalez-Gil et al., 2016;Martín et al., 2012;Narumiya et al., 2013;Pha 2009;Yang et al., 2016;Yang et al., 2017;Carballa et al., 2006;Carballa 2007b;Zhou et al., 2017;Malmborg and Magner, 2015
	Diazepam	4	Gonzalez-Gil et al., 2016;Carballa et al., 2006;Carballa et al., 2007a;Ca
	Lorazepam	0	
	Paroxetine	1	Radjenovic et al., 2009;

Table I-S8: Pair values of removal efficiency (RE) and sludge retention time (SRT) obtained from bibliography (re analysis.

Groups	Pharmaceutical compound	References
Analgesics/Anti-inflammatories (AIAPs)	**Acetaminophen**	De la Torre et al., 2015/De la Torre et al., 2015/Ghosh et al., 2009/Guerra et al., 2014/Guerra et al., 2014/Guerra et al., 2014/Jones et al., 2007/Maeng et al., 2013/Maeng et al., 2013/Maeng et al., 2013/Majewsky et al., 2011/Majewsky et al., 2011/Petrie et al., 2014/Radjenovic et al., 2007/Radjenovic et al., 2007/Radjenovic e al., 2009/Roberts and Thomas, 2006/Tambosi et al., 2010/Tambosi et al., 2010/Thiebault et al., 2017/Yan et al., 2014/Yu et al., 2006/
	Codeine	Jelic et al., 2011/Jelic et al., 2012/Sahar et al., 2011/Sahar et al., 2011/Ghosh et al., 2009/Guerra et al., 2014/Guerra et al., 2014/Guerra et al., 2014/Thiebault et al., 2017/Wick et al, 2009/

	Diclofenac	Kreuzinger et al., 2004/Kreuzinger et al., 2004/Petrie et al., 2014/Suárez et al., 2005/Thiebault et al., 2017/Bernhard et al., 2006/Bernhard et al., 2006/Bernhard et 2006/Bernhard et al., 2006/Bernhard et al., 2006/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara e al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/De la Torre et al., 2015/De la Torre et al., 2015/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Kimura et al., 2007/Kimura et al., 2007/Kimura et al., 2007/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzin et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Maeng et al., 2013/Maeng et al., 2013/Maeng et al., 2013/Majewsky et al., 2011/Majewsky et al., 2011/Radjenovic et al., 2007/Radjenovic et al., 2007/Radjenc et al., 2009/Santos et al., 2007/Santos et al., 2009/
	Ibuprofen	Radjenovic et al., 2007/Thiebault et al., 2017/Yu et al., 2006/Jelic et al., 2011/Jelic e al., 2011/Jelic et al., 2011/Jelic et al., 2012/Radjenovic et al., 2007/Radjenovic et al., 2007/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara e al., 2004/Clara et al., 2004/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/De la Torre et al., 2015/De la Torre et al., 2015/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina e al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernande Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Guerra et al., 2014/Guerra et al., 2014/Guerra et al., 2014/Jones et al., 2007/J et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss e al., 2005/Joss et al., 2005/Joss et al., 2005/Kimura et al., 2007/Kimura et al., 2007/Kimura et al., 2007/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al.,

		2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Maeng et al., 2013/Maeng et a 2013/Maeng et al., 2013/Petrie et al., 2014/Radjenovic et al., 2007/Radjenovic et al 2007/Radjenovic et al., 2009/
	Indomethacin	Roberts and Thomas, 2006/Santos et al., 2007/Santos et al., 2009/Stasinakis et al., 2013/Suárez et al., 2005/Thiebault et al., 2017/
	Ketoprofen	Vieno et al., 2005/Yan et al., 2014/Yu et al., 2006/Jelic et al., 2011/Jelic et al., 2011/ et al., 2011/Radjenovic et al., 2007/Radjenovic et al., 2007/Radjenovic et al., 2009/D la Torre et al., 2015/De la Torre et al., 2015/Jelic et al., 2011/Jelic et al., 2011/Jelic e al., 2011/Jelic et al., 2012/Kimura et al., 2007/Kimura et al., 2007/Kimura et al., 200
	Naproxen	Jelic et al., 2011/Jelic et al., 2012/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Radjenovic et al., 2007/Radjenovic et al., 2009/Thiebault et al., 2017/Vieno et 2005/Wick et al, 2009/Yan et al., 2014/De la Torre et al., 2015/De la Torre et al., 2015/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Guerra et al. 2014/Guerra et al., 2014/Guerra et al., 2014/Jelic et al., 2011/Jelic et al., 2011/Jelic al., 2011/Jelic et al., 2012/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Kimura et al., 2007/Kimura al., 2007/Kimura et al., 2007/Maeng et al., 2013/Maeng et al., 2013/
	Propyphenazone	Petrie et al., 2014/Radjenovic et al., 2007/Radjenovic et al., 2007/
Antibiotics	**Clarithromycin**	Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Sahar et al., 2011/Sahar et al., 2011/Ghosh et al., 2009/Guerra et al., 2014/Guerra et al., 2014/Guerra et al., 2014/
	Ofloxacin	Radjenovic et al., 2007/Radjenovic et al., 2007/Radjenovic et al., 2009/Stasinakis et 2013/Suárez et al., 2005/Tambosi et al., 2010/Tambosi et al., 2010/Thiebault et al., 2017/Vieno et al., 2005/Guerra et al., 2014/Guerra et al., 2014/Guerra et al., 2014/J et al., 2012/
	Sulfadiazine	Petrie et al., 2014/Radjenovic et al., 2009/Wick et al, 2009/Radjenovic et al., 2007/Radjenovic et al., 2007/

	Sulfamethazine	Radjenovic et al., 2009/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et al. 2012/Radjenovic et al., 2009/
	Sulfamethoxazole	Vieno et al., 2005/Wick et al, 2009/Xia et al., 2012/Xia et al., 2012/Xia et al., 2012/X et al., 2012/Yan et al., 2014/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Li an Zhang, 2011/Sahar et al., 2011/Yan et al., 2014/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/De la Torre et al., 2015/De la Torre et a 2015/Ghosh et al., 2009/Göbel et al., 2007/Göbel et al., 2007/Joss et al., 2005/Joss al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et a 2005/Joss et al., 2005/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et a 2004/Li and Zhang, 2011/Majewsky et al., 2011/Majewsky et al., 2011/
	Trimethoprim	Radjenovic et al., 2007/Radjenovic et al., 2007/Radjenovic et al., 2009/Sahar et al., 2011/Sahar et al., 2011/Tambosi et al., 2010/Tambosi et al., 2010/Xia et al., 2012/X al., 2012/Xia et al., 2012/Xia et al., 2012/Xu et al., 2007/Yan et al., 2014/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Ghosh et al., 2009/Göbel et al 2007/
Beta-Blockers	**Atenolol**	Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/
	Metoprolol	Jelic et al., 2012/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Radjenovic et al. 2007/Radjenovic et al., 2009/Thiebault et al., 2017/Vieno et al., 2005/Wick et al, 20
	Propranolol	Xu et al., 2007/Yan et al., 2014/Radjenovic et al., 2007/Radjenovic et al., 2007/De la Torre et al., 2015/
	Sotalol	Radjenovic et al., 2009/Wick et al, 2009/Radjenovic et al., 2007/Radjenovic et al., 2007/Radjenovic et al., 2009/Jelic et al., 2011/Jelic et al., 2011/
Diuretics	**Furosemide**	Maeng et al., 2013/Maeng et al., 2013/Radjenovic et al., 2007/Radjenovic et al., 200
	Hydrochlorothiazide	Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2005a/Clara et al., 2005a/

Lipid-regulators	**Bezafibrate**	Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et 2005b/Clara et al., 2005b/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et 2012/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzin et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Maeng et al., 2013/Maeng et al., 2013/Maeng et al., 2013/Petrie et al., 2014/Radjenovic et al., 2007/Radjenovic et al., 2007/Radjenovic al., 2009/Vieno et al., 2005/Yan et al., 2014/Bernhard et al., 2006/Bernhard et al., 2006/Bernhard et al., 2006/Bernhard et al., 2006/Bernhard et al., 2006/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/
	Fenofibrate	Maeng et al., 2013/Radjenovic et al., 2007/Radjenovic et al., 2007/
	Gemfibrozil	Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Radjenovic et al., 2007/Radjenovi al., 2007/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Clara et al., 2004/Cla et al., 2004/Clara et al., 2004/
Psychiatric Medications	**Carbamazepine**	Clara et al., 2005a/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/ la Torre et al., 2015/De la Torre et al., 2015/Jelic et al., 2011/Jelic et al., 2011/Jelic e al., 2011/Jelic et al., 2012/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Joss et al., 2005/Kreuzinger al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Kreuzinger et al., 2004/Maeng et al., 2013/Maeng et a 2013/Maeng et al., 2013/Petrie et al., 2014/Radjenovic et al., 2007/Radjenovic et al 2009/Suárez et al., 2005/Thiebault et al., 2017/Wick et al, 2009/Xue et al., 2010/Yar al., 2014/Ghosh et al., 2009/Göbel et al., 2007/Göbel et al., 2007/Jelic et al., 2011/J et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Sahar et al., 2011/Sahar et al., 2011/Ghosh et al., 2009/Guerra et al., 2014/Guerra et al., 2014/Guerra et al., 2014/Thiebault et al., 2017/Wick et al, 2009/Yadav et al., 2019/Yadav et al., 2019/Yadav et al., 2019/Yadav et al., 2019/Jelic et al., 2011/

	Diazepam	Clara et al., 2004/Clara et al., 2004/Clara et al., 2005a/Clara et al., 2005a/Clara et al. 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/Clara et al., 2005a/
	Lorazepam	Vieno et al., 2005/Wick et al, 2009/Yan et al., 2014/De la Torre et al., 2015/
	Paroxetine	Radjenovic et al., 2007/Radjenovic et al., 2007/

Table I-S9: Pair values of removal efficiency (RE) and hydraulic retention time (HRT) obtained from bibliography analysis.

Groups	Pharmaceutical compound	References
Analgesics/Anti-inflammatories (AIAPs)	**Acetaminophen**	De la Torre et al., 2015/Guerra et al., 2014/Guerra et al., 2014/Guerra et al., 2014/Majewsky et al., 2011/Majewsky et al., 2011/Petrie et al., 2014/Radjenovic 2009/Tambosi et al., 2010/Tambosi et al., 2010/Thiebault et al., 2017/
	Codeine	Jelic et al., 2011/Jelic et al., 2012/Sahar et al., 2011/Sahar et al., 2011/Guerra et 2014/Guerra et al., 2014/Guerra et al., 2014/Thiebault et al., 2017/
	Diclofenac	Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Petrie et al., 2014/Thiebault e 2017/Bernhard et al., 2006/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/De la Torre et al., 2015/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Kimura et al., 2007/Majewsky et al., 2011/Majewsky et al., 2011/

	Ibuprofen	Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Thiebault et a 2017/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Bernha al., 2006/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005 la Torre et al., 2015/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernande Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Guerra et al., 2014/
	Indomethacin	Guerra et al., 2014/Guerra et al., 2014/Kimura et al., 2007/Martínez-Alcalá et al.
	Ketoprofen	Petrie et al., 2014/Radjenovic et al., 2009/Stasinakis et al., 2013/Thiebault et al., 2017/Yan et al., 2014/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Radjenc al., 2009/De la Torre et al., 2015/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 20
	Naproxen	Xue et al., 2010/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Radjenovic et al., 2009/Thiebault et al., 2017/Yan et al., 2014/De la Torre et al., 2015/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernande Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Guerra et al., 2014/Guerra 2014/Guerra et al., 2014/Jelic et al., 2011/
	Propyphenazone	Stasinakis et al., 2013/
Antibiotics	**Clarithromycin**	Sahar et al., 2011/Sahar et al., 2011/Guerra et al., 2014/Guerra et al., 2014/Guer al., 2014/Thiebault et al., 2017/
	Ofloxacin	Jelic et al., 2012/Kimura et al., 2007/Martínez-Alcalá et al., 2017/Petrie et al., 2014/Radjenovic et al., 2009/Stasinakis et al., 2013/Tambosi et al., 2010/
	Sulfadiazine	Jia et al., 2012/
	Sulfamethazine	Petrie et al., 2014/Radjenovic et al., 2009/Yan et al., 2014/De la Torre et al., 2015/Petrie et al., 2014/

	Sulfamethoxazole	Radjenovic et al., 2009/Radjenovic et al., 2009/Jelic et al., 2011/Jelic et al., 2011/ al., 2011/Jelic et al., 2012/Radjenovic et al., 2009/Yan et al., 2014/Jelic et al., 201 et al., 2011/Jelic et al., 2011/Sahar et al., 2011/Yan et al., 2014/
	Trimethoprim	Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/De la et al., 2015/Majewsky et al., 2011/Majewsky et al., 2011/Radjenovic et al., 2009/ et al., 2011/Sahar et al., 2011/Tambosi et al., 2010/Tambosi et al., 2010/Yan et a 2014/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernande Fontaina et al., 2012/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et al.,
Beta-Blockers	**Atenolol**	Clara et al., 2005b/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Petrie et al., 2014/
	Metoprolol	Jelic et al., 2012/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Radjenovic et 2009/Thiebault et al., 2017/
	Propranolol	Jia et al., 2012/Petrie et al., 2014/Radjenovic et al., 2009/
	Sotalol	Yan et al., 2014/De la Torre et al., 2015/Petrie et al., 2014/Radjenovic et al., 2009/Radjenovic et al., 2009/
Diuretics	**Furosemide**	Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/
	Hydrochlorothiazide	Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/
Lipid-regulators	**Bezafibrate**	Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara 2005b/Clara et al., 2005b/De la Torre et al., 2015/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Martínez-Alcalá et al., 2017/Petrie et al., 2014/Radjenovic et al., 2009/Thiebault et al., 2017/
	Fenofibrate	Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/

	Gemfibrozil	Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara et al., 2005b/Clara 2005b/
Psychiatric Medications	**Carbamazepine**	Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2012/Sahar et al., 2011/Sahar et al., 2011/Guerra et al., 2014/Guerra et al., 2014/Guerra et al., 2014/Thiebault et al., 2017/Yadav et al., 2019/Yadav et al., 2019/Yadav et al., 2019/Yadav et al., 2019/Jelic et al., 2011/Jelic et al., 2011/Jelic et al., 2011/Petrie 2014/Thiebault et al., 2017/Bernhard et al., 2006/
	Diazepam	Jelic et al., 2012/Kimura et al., 2007/Majewsky et al., 2011/Majewsky et al., 2011/Martínez-Alcalá et al., 2017/
	Lorazepam	De la Torre et al., 2015/Fernandez-Fontaina et al., 2012/Fernandez-Fontaina et a 2012/Fernandez-Fontaina et al., 2012/
	Paroxetine	

II. OBJETIVOS

El **objetivo principal de esta Tesis Doctoral ha sido** estudiar la presencia, el balance másico y la eficiencia de eliminación de 27 compuestos farmacéuticos activos (8 analgésicos/antiinflamatorios, 6 antibióticos, 4 beta-bloqueantes, 2 diuréticos, 3 reguladores lipídicos y 4 medicamentos psiquiátricos) tanto en línea de aguas como en línea de fangos en una planta piloto que trata agua residual urbana de la EDAR Murcia Este. En línea de aguas se operó con un sistema convencional A^2O y otro avanzado A^2O-IFAS en tres fases experimentales y en línea de fangos con un sistema avanzado de digestión bifásica.

Para alcanzar el objetivo principal de la investigación, se han abordado los siguientes objetivos específicos:

1. Análisis de la influencia de las diferentes condiciones operacionales propuestas para Fase I y II en el sistema A^2O y la influencia de la adicción de soporte plástico en Fase III sobre la capacidad de eliminación de materia orgánica y nutrientes, así como el estudio de la digestión bifásica a diferentes tiempos de retención hidráulica (TRH) propuestos en Fase I y II sobre la eliminación de materia orgánica volátil y producción de biogás en línea de fangos.

2. Estudios microbiológicos sobre cuantificación, diversidad y función de las comunidades microbianas (bacterias filamentosas, hongos y arqueas) en las diferentes Fases de estudio tanto en el biorreactor A^2O de la línea de aguas como en los dos digestores de la línea de fangos. La finalidad principal fue encontrar correlaciones entre los parámetros operacionales, diversidad microbiana y ratios de eliminación de los compuestos farmacéuticos en los tres biorreactores estudiados.

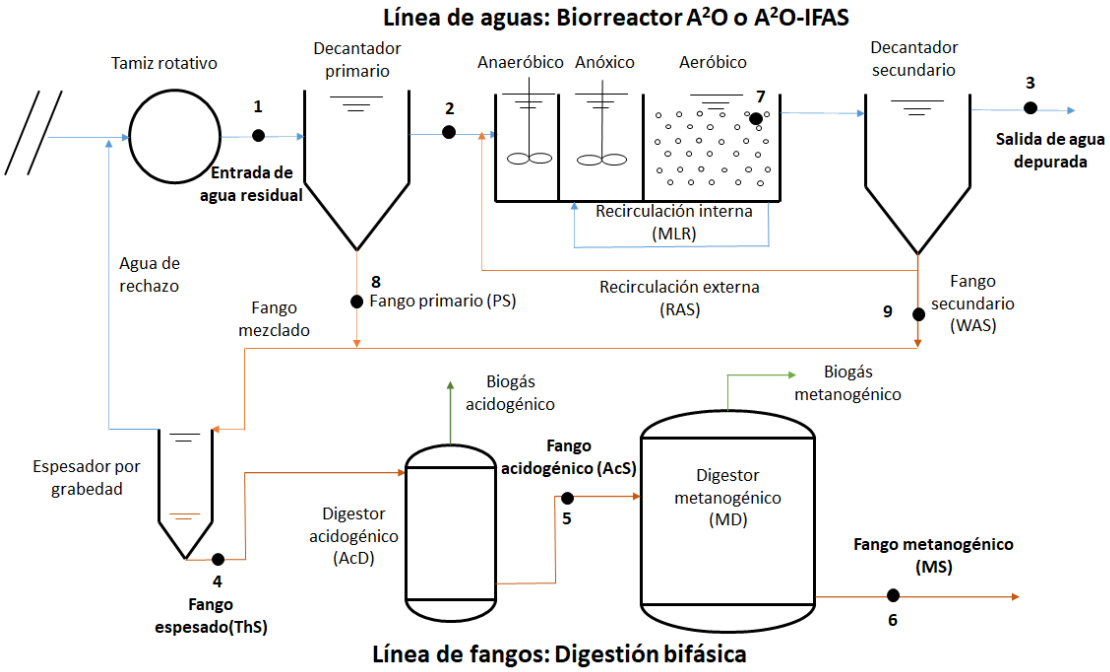
3. Análisis de las concentraciones en entrada y salida de los 27 compuestos farmacéuticos tanto en línea de aguas como en línea de fangos para adquirir conocimientos sobre la capacidad de eliminación/remoción de estos fármacos en diferentes sistemas de depuración (A^2O, A^2O-IFAS o digestión bifásica) y bajo diferentes parámetros operacionales.

III. MATERIALES Y MÉTODOS

1. Descripción general de la planta piloto de tratamiento de aguas residuales urbanas

La planta piloto utilizada en las investigaciones de la presente Tesis Doctoral es una réplica de la EDAR Murcia Este, y se encuentra ubicada dentro del recinto de estas instalaciones. El diagrama de flujo de la planta piloto se muestra en la Figura III-1. La planta piloto trata el agua residual urbana proveniente después del proceso de pretratamiento y desbaste de la EDAR Murcia Este, en el cual los equipos unitarios de la línea de aguas (flechas en azul) están diseñados para tratar un caudal medio de 4 m^3d^{-1} y un caudal punta máximo de 6 m^3d^{-1}, así como la línea de fangos (flechas en marrón) está diseñada para tratar el fango primario y secundario producidos por la línea de aguas.

Figura III-1. Diagrama de flujo de la planta piloto FLUSER



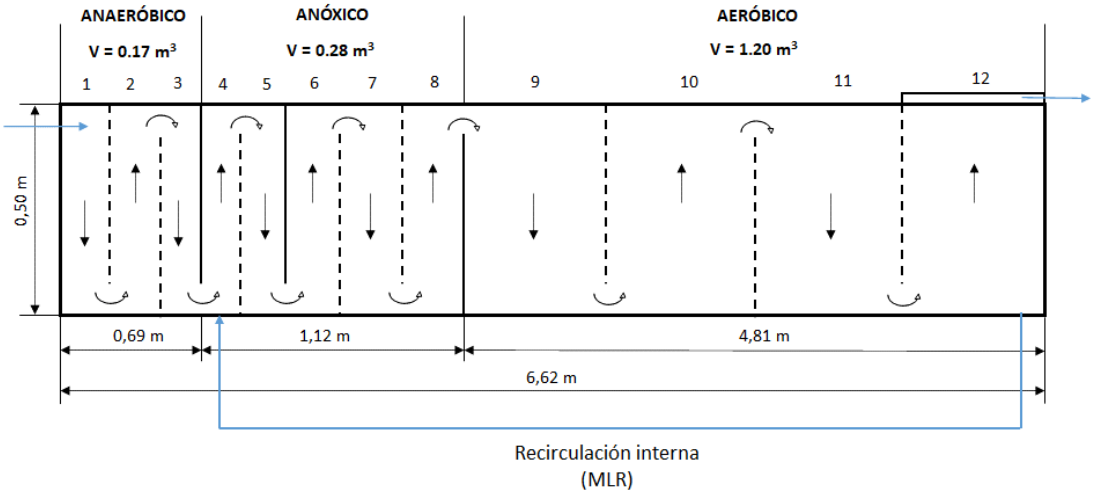
El agua bruta se introduce primeramente por un tamiz rotativo auto limpiante con un paso de 0.5 mm, el agua posteriormente pasa al decantador primario (diámetro 0.75 m) donde se obtiene agua decantada y fango primario. El agua decantada se introduce al reactor biológico diseñado para eliminar nitrógeno y fósforo con la configuración A^2O (anaeróbico, anóxico y aeróbico). La configuración y el esquema del reactor biológico se representa en la Figura III-2, mientras que el volumen de cada compartimento y los tiempos de retención hidráulicos (TRH) son mostrados en la Tabla III-1. El biorreactor está dividido en 12 cámaras para producir una mejor mezcla del fango activo y operar en flujo pistón;

las tres primeras cámaras son anaeróbicas con un volumen total de 0.17 m^3, las cinco siguientes son anóxicas con un volumen total de 0.28 m^3 y las cuatro últimas son aeróbicas con un volumen total de 1.20 m^3. La cámara anóxica 4 recibe una corriente de recirculación de fango activo con nitratos (recirculación interna, en inglés "Mixed-Liquor Recycle, MLR") proveniente de la cámara aeróbica 12. El biorreactor tiene un volumen total de 1.655 m^3 con un TRH de 9.9 horas para el caudal medio y TRH de 6.6 horas para el caudal punta máximo. Posteriormente, el fango activo pasa al decantador secundario (diámetro de 0.8 m) donde se produce el efluente depurado y fango secundario. Una parte del fango secundario es recirculado a la cámara anaeróbica 1 mediante la recirculación externa (RAS) y otra parte es enviada al espesador por gravedad.

Tabla III-1. Volumen, porcentaje de volumen y tiempos de retención hidráulico (TRH) de cada compartimento del biorreactor.

Zona	V_{zona}, m^3	%V total	TRH diseño, h	TRH punta, h
Anaerobia	0.17	10.5	1.0	0.7
Anóxica	0.28	16.9	1.7	1.1
Aerobia	1.20	72.7	7.2	4.8
Total	1.65	100	**9.9**	**6.6**

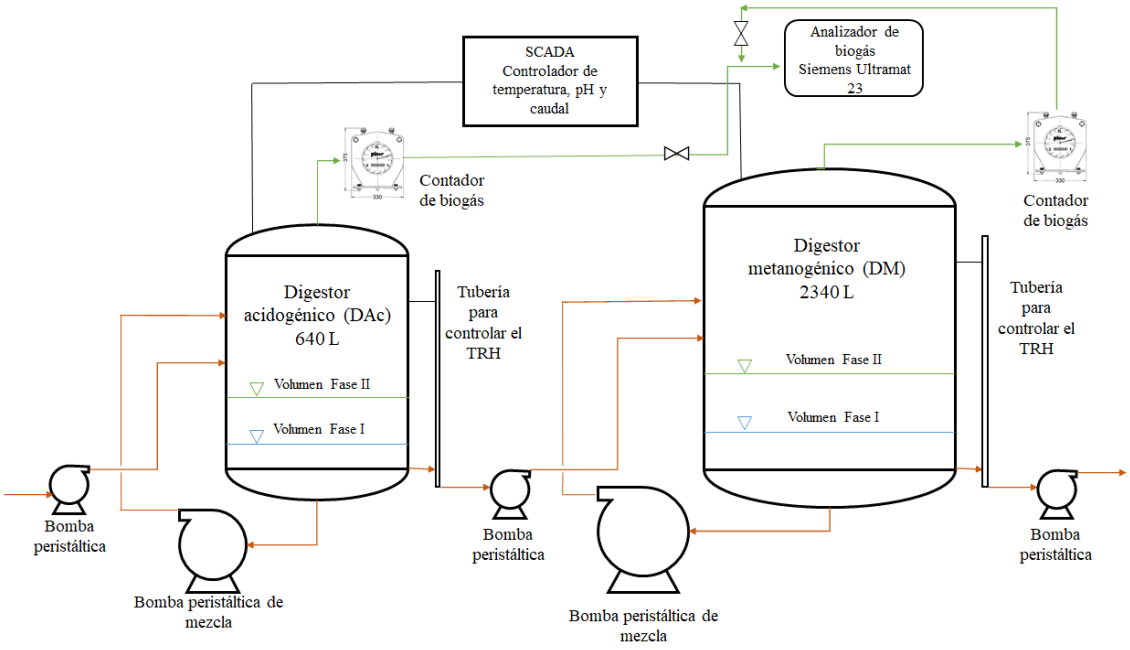
Figura III-2. Esquema y dimensiones del reactor biológico A^2O.



La línea de fangos se inicia con la mezcla y espesamiento del fango primario y secundario en el espesador por gravedad (216 L de volumen, 0.5 m de diámetro y una altura de 1.45 m). El sobrenadante del espesador vuelve a cabecera y el fango espesado (ThS) entra en el digestor acidogénico (AcD) donde se producen los procesos de hidrólisis y

acidogénesis de la digestión anaeróbica. Posteriormente, el fango de salida del digestor acidogénico (AcS) entra en el digestor metanogénico (DM) donde se produce los procesos finales de la digestión anaeróbica, acetogénesis y metanogénesis. En la Figura III-3 se muestra el diagrama de flujo detallado de la digestión bifásica; el digestor primario o acidogénico tiene un volumen de 640 L y el digestor secundario o metanogénico un volumen de 2340 L. Estos están controlados térmicamente por una camisa térmica a 35 ºC y la mezcla de fango en el interior se realiza mediante recirculación por bombas peristálticas. Dos medidores de gas tipo tambor (TG3 plástico, Ritter Company) miden el caudal de biogás y el analizador Siemens Ultramat 23 es conectado para medir la composición del biogás. Toda la planta piloto está controlada por un sistema informático de control y almacenamiento de datos ("Supervisory Control and Data Acquisition, SCADA") para controlar los parámetros operacionales del sistema.

Figura III-3. Diagrama de flujo de la digestión bifásica



2. Plan de investigación y parámetros operacionales principales

Para dar una estabilidad necesaria a los procesos y dar respuestas a los objetivos planteados, se han definido tres fases de estudios. Los principales parámetros operacionales seleccionados para definir las fases de investigación fueron: diferente concentración de fango activo (MLSS) y carga másica (en inglés "food-to-microorganism ratio", F/M) en

línea de aguas, el uso o no de material relleno plástico para operar con un sistema IFAS en los biorreactores y diferentes TRH en los digestores de la digestión bifásica.

En la Tabla III-2 se muestran los principales parámetros operacionales y la duración de las tres fases estudiadas en la presente investigación junto con los periodos de puesta en marcha necesarios para el arranque de cada fase.

Tabla III-2. Fases de investigación y parámetros operacionales principales

Fases de estudio	**Duración (días)**	**Estado del biorreactor**	**F/M ratio (kg BOD_5 kg $MLVSS^{-1}$ d^{-1})**	**MLSS (mg L^{-1})**	**TRH DAc (d)**	**TRH DM (d)**
01	68	Puesta en marcha	0.30-0.35	2500-3000	2	12
I	**104**	**Sin relleno**	**0.34**	**2600**	**2**	**12**
02	30	Fase de transición	0.20-0.25	4500-5000	5	24
II	**105**	**Sin relleno**	**0.21**	**5000**	**5**	**24**
03	92	Puesta en marcha con relleno	0.30-0.40	3000-2000	-	-
III	**105**	**Con relleno**	**0.41**	**1800**	**-**	**-**

A continuación, se describe el desarrollo y procedimiento de las diferentes fases de estudio de la presente investigación:

- **01**: La planta piloto se puso en marcha el 20 de mayo de 2016 mediante la inoculación del biorreactor A^2O con fango activo (1000 L) y mediante la inoculación de los digestores con fango digerido (200 L) procedente de la EDAR Murcia Este. Desde el 1 de junio, el biorreactor A^2O de la línea de aguas empezó a operar en estado estacionario con una concentración de fango activo entre 2500-3000 mg L^{-1} y una carga másica (F/M) de entre 0.30-0.35 kg BOD_5 kg $MLVSS^{-1}$ d^{-1}. De esta forma, se produjo un caudal regular de fango espesado de 41 ± 9 L d^{-1} y por consiguiente los digestores empezaron a operar en estado estacionario con TRH constante de 2 días para el DAc y 12 días para el DM.

- **Fase I:** El comienzo de la Fase I se realiza con la primera recogida de muestras para el análisis de los 27 PhACs seleccionados, así como muestras del fango activo y del fango digerido para la caracterización microbiológica. La Fase I comenzó el 27 de julio de 2016 con las condiciones operacionales seleccionadas para la línea de aguas y la línea de fangos anteriormente descritas (Tabla III-2).

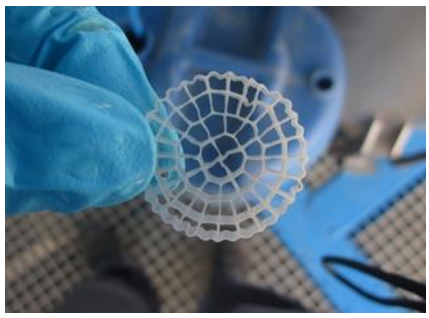
- **02:** Periodo de transición para la puesta en marcha de las nuevas condiciones operacionales seleccionadas para la Fase II (Tabla III-2).

- **Fase II:** La Fase II comenzó el 14 de diciembre de 2016 con diferentes parámetros operacionales para el biorreactor de la línea de aguas y con diferentes TRH para los digestores de la línea de fangos (Tabla III-2). Los datos obtenidos de la Fase I y II han sido descritos, estudiados y comparados en los tres primeros capítulos de la presente Tesis Doctoral.

- **03:** Puesta en marcha del reactor biológico A^2O-IFAS con el material de relleno plástico AnoxKaldnes K5 (características descritas en la Tabla III-3). Para ello, las cuatro cámaras aeróbicas del biorreactor son rellenadas con 0.6 m^3 de relleno plástico AnoxKaldnes K5, ocupando así el 50% del volumen aeróbico y un 36% el volumen total del biorreactor. La puesta en marcha y la fase de estabilización del sistema A^2O-IFAS tienen una duración de 92 días (desde 02/05/17 hasta el 02/08/17). A partir del 1 de junio comenzó la fase de estabilización del sistema al operar en estado estacionario con las condiciones operacionales seleccionadas para la Fase III (Tabla III-2).

- **Fase III:** La Fase III comenzó el 2 de agosto con las condiciones operacionales descritas en la Tabla III-2 y con la primera recogida de muestras para el análisis de fármacos. En el cuarto capítulo se estudian y analizan los datos de rendimiento de eliminación de materia orgánica, nutrientes y fármacos de la Fase III que opera con un sistema avanzado A^2O-IFAS, estos datos fueron comparados con los datos obtenidos en el sistema convencional A^2O de las Fase I y II.

Tabla III-3. Características del soporte de relleno AnoxKaldnes K5

Características	AnoxKaldnes K5	Foto del soporte
Material	Polietileno de alta densidad	
Forma	Cilíndrica	
Densidad, kg L^{-1}	0.95	
Densidad aparente, Kg m^{-3}	118	
Área específica, m^2 m^{-3}	800	
Diámetro nominal, mm	25	
Altura nominal, mm	3.5	
Número de soportes por m^3	331000	

3. Programa de muestreos para el análisis de los parámetros físico-químicos, el análisis de los compuestos farmacéuticos y el análisis de la comunidad microbiana

Para el análisis de los parámetros físico-químicos que describen y controlan el proceso de depuración de agua residual urbana y de digestión de fangos, se han realizado recogidas de muestras tres veces por semana en todos los puntos (del 1 al 9) señalados en el diagrama de flujo presentado en la Figura III-1.

En la Tabla III-4 se muestran los puntos de toma de muestras para el análisis de la comunidad microbiana y de los compuestos farmacéuticos tanto en línea de aguas como en línea de fangos, y en la Figura III-4 se muestra un esquema por fases de los días seleccionados para la recogida de muestras necesarias para el análisis de la comunidad microbiana (micro) y el análisis de los compuestos farmacéuticos (PhACs). Para el análisis de los compuestos farmacéuticos en la Fase I, se tomó un total de cuatro muestras (n=4) por cada punto de muestreo (Tabla III-4 y Figura III-1), tanto en línea de aguas como en línea de fangos a principio de fase y final de fase (entre semana y fin de semana). Debido a las intensas lluvias producidas el fin de semana después de la primera recogida de muestras de la Fase II (14-12-2016) no se realizó la toma de muestras programada para el día 18-12-2016, esta se realizó 35 días después coincidiendo con la recogida de muestra de microbiología, además se realizó otra toma de muestra ese fin de semana. Por lo tanto, en Fase II se recogió un total de 5 muestras (n=5) por cada punto de muestreo a lo largo de la fase, tanto en línea de aguas como en línea de fangos. Finalmente, para la Fase III se recogió un total de 6 muestras (n=6) por cada punto de muestreo de la línea de aguas, entre semana y fin de semana las cuatro primeras muestras y entre semana las dos últimas (Figura III-4). Por otra parte, la toma de muestras para el análisis microbiológico en Fase I y II se realizó

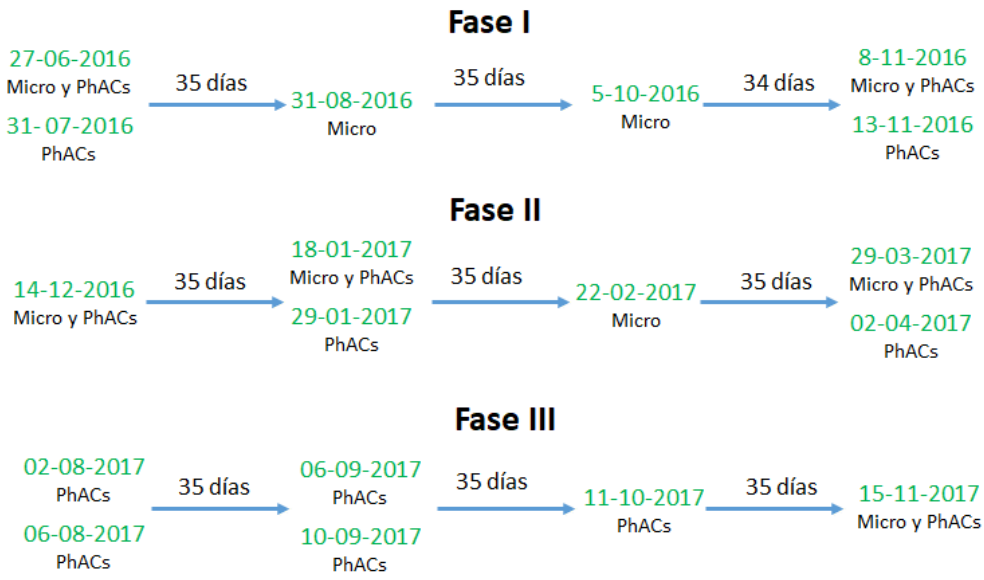
de forma distribuida cada 35 días desde el inicio hasta el final de cada fase, se tomaron muestras (200 mL) tanto en línea de aguas con en línea de fangos en los puntos indicados en la Tabla III-4.

Para el análisis de los compuestos farmacéuticos en línea de aguas se tomaron 500 mL en una botella de plástico PET ámbar de una muestra integrada de 6 L recogida durante 24 horas por cada punto de muestreo; la muestra fue congelada a -20 ºC para ser posteriormente analizada. Entre la recogida de muestra de entrada y salida del agua residual se tuvo en cuenta el TRH total de la línea de aguas (12 horas) para disminuir posibles fluctuaciones de la concentración de fármacos durante el día. Por otra parte, para el análisis de los compuestos farmacéuticos en línea de fangos se tomaron 500 mL en botella de plástico PET ámbar de una muestra compuesta de 6 L de fango recogida durante el día en cada punto de toma de muestra. Igualmente, la muestra fue congelada a -20ºC para su posterior análisis. Todas las muestras fueron tomadas en periodos sin lluvias para evitar la dilución de la muestra.

Tabla III-4: Puntos de toma de muestras para los análisis de comunidad microbiana y de los compuestos farmacéuticos (PhACs). Los puntos pertenecen al diagrama de flujo de la Figura III-1.

Microbiología		**Compuestos farmacéuticos (PhACs)**	
Línea de aguas	***Línea de fangos***	***Línea de aguas***	***Línea de fangos***
Reactor biológico, cámara aeróbica 11 (punto 7)	Salida Espesador (punto 4)	Entrada decantación primaria (punto 2)	Salida Espesador (punto 4)
	Salida Digestor primario (punto 5)	Salida decantación secundaria (punto 3)	Salida Digestor primario (punto 5)
	Salida digestor secundario (punto 6)		Salida digestor secundario (punto 6)

Figura III-4. Fechas de toma de muestras para los análisis de la comunidad microbiana (Micro) y de los compuestos farmacéuticos (PhACs) de las tres fases de experimentación.



4. Análisis de los parámetros fisicoquímicos de la línea de aguas y línea de fangos

En línea de aguas, los análisis de la demanda química de oxígeno (DQO), nitrógeno total (TN), $N\text{-}NO_3^-$, $N\text{-}NH_4^+$ y fósforo total (TP) fueron realizados mediante los kits Merck Spectroquant® kits (Darmstadt, Alemania), mientras que la concentración de fango activo (MLSS), fango activo volátil (MLVSS), solidos suspensos totales (TSS) y demanda biológica de oxígeno (BOD_5), fueron obtenidos de acuerdo con la "American Public Health Association" (APHA) "Standard Methods for the Examination of Water and Wastewater" (SM 2540 para MLSS, MLVSS y TSS; SM 5210B para BOD_5) (Baird and Bridgewater, 2017). La concentración de biomasa adherida a los soportes plásticos (BFSS) se ha calculado siguiendo el procedimiento anteriormente publicado por Leyva-Díaz and Poyatos (2015) y Monteoliva-García et al. (2019). Resumidamente, se recogen doce soportes plásticos de la forma más representativa posible del biorreactor (tres por cada cámara aeróbica), se introducen en 50 mL de agua destilada con el tensioactivo Tween 80 (1/1000 de dilución), son sonicados durante 15 minutos y centrifugados durante 20 minutos a 3000 rpm. Una vez la biomasa es separada de los soportes plásticos y suspendida en los 50 mL de agua, la concentración de sólidos adheridos (BFSS) es analizada de igual forma que la concentración de MLSS aplicándole el número total de soportes en el biorreactor (331 unidades L^{-1}) por volumen total del biorreactor (1655 L).

En línea de fangos, dos medidores de gas tipo tambor (TG3 plástico, Ritter Company) midieron la producción de biogás; este parámetro es controlado y almacenado diariamente por el sistema de control SCADA, que también controla diariamente la temperatura de los digestores (35ºC) y el pH. La composición de biogás (CH_4, CO_2 and O_2) se analizó tres veces por semana mediante el analizador Siemens Ultramat 23 y una vez al mes por el analizador portátil Geotechnical Instrument Ltd, GA5000 para verificar el análisis diario. Los sólidos totales (TS) y sólidos volátiles (VS) del fango fueron medidos según Standard Methods (SM 2540) (Baird and Bridgewater, 2017). La eliminación de los sólidos volátiles (VSR) se realizó mediante un balance de sólidos volátiles al digestor (Bhattacharya et al., 1996). Finalmente, la concentración de los compuestos ácidos grasos volátiles (VFA) y la alcalinidad (ALK) se midieron mediante valoración según el método propuesto por DiLallo and Albertson (1961).

5. Análisis de los compuestos farmacéuticamente activos (PhACs) tanto en línea de aguas como en línea de fangos

Las muestras congeladas a -20 ºC fueron enviadas al Instituto de Evaluación Ambiental e Investigación del Agua (IDAEA-CSIC) en Barcelona (IDAEA-CSIC) para el análisis de los compuestos farmacéuticos. A continuación, se describe la metodología utilizada.

5.1. Compuestos químicos, reactivos y soluciones utilizadas

Los patrones de referencia analíticos de alta pureza (>90%) (acetaminofén, atenolol, bezafibrato, carbamazepina, claritromicina, codeína, diazepam, diclofenaco, fenofibrato, furosemida, gemfibrozil, hydroclorotiazida, ibuprofeno, indometacina, ketoprofeno, lorazepam, metoprolol, naproxeno, ofloxacin, paroxetina, propanolol, propifenazona, sotalol, sulfadiazina, sulfametazina, sulfametoxazol, trimetoprim) se adquirieron de Sigma Aldrich (St. Luis, MO, EE. UU.). Los compuestos marcados con isótopos (acetaminophen-d4, bezafibrate-d4, carbamazepine-d10, codeine-d3, diazepam-d5, diclofenac-d4, furosemide-d5, fenofibrate-d6, gemfibrozil-d6, ketoprofen-d3, indomethacin-d4, lorazepam-d4, ofloxacine-d3, metoprolol-d7, naproxen-d3, paroxetine-d4, propranolol-d7, sotalol-d6, sulfamethoxazole-d4, sulfadiazine-d4, sulfamethazine-d4) se adquirieron en Cerilliant, Alsachim-Graffenstakirden Francia) o Santa Cruz Biotechnology (Dallas, TX, EE. UU.). El número CAS, fórmulas moleculares, peso molecular y otras propiedades

relevantes de todos los compuestos diana son mostrados en (Gallardo-Altamirano et al., (2019, 2018).

Se prepararon soluciones madre estándar individuales (concentración de 1000 µg mL^{-1}) en metanol al 100% o en DMSO al 100%, según la solubilidad de cada compuesto. Se preparó una mezcla de soluciones de trabajo que contenía todos los analitos antes mencionados y los compuestos marcados isotópicamente (1 µg mL^{-1}), para propósitos de análisis y calibración, diluyendo volúmenes adecuados de las soluciones madre individuales con MeOH. Todas las soluciones se almacenaron a -20 °C.

5.2. Preparación y extracción de las muestras de agua residual y de fangos

Para las muestras de la línea de aguas, se realizó un pretratamiento siguiendo las instrucciones descritas en los métodos descritos por Gros et al. (2009) y López-Serna et al. (2010). Abreviadamente, las muestras de agua se filtraron a través de filtros de fibra de vidrio de 1 µm (Whatman, Fairfield, Connecticut, EE. UU.), seguido de filtración a través de filtros de membrana de nailon de 0,45 µm (Teknokroma, Barcelona, España). Finalmente, se añadieron 10 µL de una disolución estándar de compuestos mezclados a una concentración de 100 ng mL^{-1} en un volumen de 10 mL de muestra para el control del compuesto patrón añadido y para una calibración interna estándar. La preconcentración de muestras de aguas residuales y separación cromatográfica se realizó en un dispositivo automatizado en línea SPE-LC Symbiosis™ Pico (Spark Holland, Emmen, Países Bajos) en el modo on-line SPE utilizando cartuchos SPE HySphere Resin GP 10 (polidivinil-benceno, tamaño de partícula de 5 a 15 µm) (Spark Holland, Emmen, Países Bajos). El cartucho se acondicionó con 2 ml de MeOH y 2 ml de agua, a un caudal de 5 ml min^{-1}, luego se cargaron 2,5 ml de muestra en el cartucho a un caudal de 1 ml min^{-1} para mejorar la retención de los analitos en el solvente. Antes de la elución, el cartucho se enjuagó con 1 ml de agua de grado HPLC a una velocidad de flujo de 5 ml min^{-1} para completar la transferencia de la muestra y eliminar las interferencias. Finalmente, los analitos se eluyeron mediante el método "estándar", en el que el gradiente cromatográfico completo pasa a través del cartucho SPE antes de ser conducido a la columna LC. Durante la elución, se colocó un nuevo cartucho en modo de preconcentración para la siguiente muestra. De esta manera, la elución de una muestra y la preconcentración de una segunda muestra se llevaron a cabo simultáneamente. Todo el gradiente eludido pasó a través de la columna LC para una separación cromatográfica.

Para la extracción y el análisis de muestras de fangos se aplicaron métodos previamente publicados por Gago-Ferrero et al. (2015) y Jelić et al. (2009) respectivamente, con algunas modificaciones. Resumidamente, se liofilizaron 500 ml de muestras de lodo durante aproximadamente 10 días hasta peso constante. Luego, se colocaron 0,5 g del fango liofilizado y tamizado en un tubo Falcon de 50 ml y se añadieron 50 ng de la correspondiente disolución mezcla de compuestos patrón, y se mantuvo en contacto durante la noche en una campana extractora. Se agregaron 5 mL de solvente de extracción (MeOH-HPLC agua 1:2 (v/v)) y el tubo se agitó durante 1 min. Luego se extrajo la muestra mediante baño ultrasónico durante 10 min. Después de la primera extracción, el tubo se centrifugó durante 10 min (4000 rpm, 4 ° C) y el sobrenadante se recogió en un tubo de ensayo de vidrio de 16 mL. Este procedimiento se repitió dos veces más, pero la última vez se utilizaron como disolvente de extracción 5 ml de ácido fórmico al 0,1% en MeOH/agua 1:1 (v/v). En total, se recogieron aproximadamente 15 ml de sobrenadante. Luego, el extracto se evaporó a menos de 10 mL bajo un suave vapor de nitrógeno a 24 ºC usando un TurboVap® LV (Biotage AB, Uppsala, Suecia) para reducir la cantidad de metanol para el siguiente paso SPE (extracción en fase sólida). El extracto se diluyó en 100 ml de agua de HPLC para asegurar una concentración de disolvente inferior al 1%. El volumen total de las soluciones acuosas se filtró a través de una membrana de nailon con un tamaño de poro de 0,45 µm, 47 mm de diámetro y se transfirió al procedimiento SPE utilizando cartuchos Oasis® HLB (60 mg, 3 ml, Waters Corporation - Milford, MA, EE. UU.). Para el SPE, los cartuchos se acondicionaron previamente con 5 mL de EtAc y 5 mL de MeOH, y se equilibraron con 5 mL de agua ultrapura, y luego la muestra se cargó en los cartuchos a un caudal de 1 mL min^{-1}. Se realizó una etapa de lavado con 2x3 mL de agua ultrapura y, antes de la elución, los cartuchos se secaron bajo aspiración al vacío durante 30 min. Los analitos se eluyeron con 3x3 ml de EtAc: MeOH (1:1, v/v). El extracto se evaporó a sequedad bajo una suave corriente de nitrógeno y se reconstituyó con 1 mL de H2O/MeOH (90:10) y luego se filtró a viales de 2 mL usando filtros de jeringa de politetrafluoroetileno (PTFE) de 0,22 µm para análisis UPLC-MS/MS.

5.3. Separación HPLC y análisis de las muestras de agua mediante ESI-QqLIT MS/MS

El gradiente eludido pasó a través de una fase inversa Purospher Star RP-18 con tapa terminal (125 mm × 2,0 mm, tamaño de partícula 5 µm) (Merck, Darmstadt, Alemania) para la separación de compuestos. Para la detección por espectrometría de masas,

Symbiosis™ Pico operó en modo LC de extracción conectado en serie con un espectrómetro de masas de trampa de iones lineales cuadrupolo triple híbrido 4000 QTrap (QqLIT), equipado con una fuente de pulverización de iones Turbo (Applied Biosystems-Sciex, Foster City, California, Estados Unidos).

La elución de los analitos se realizó siguiendo las instrucciones descritas por Gros et al. (2009). Así, para el modo de ionización positiva (PI), se aplicó un gradiente de ACN/ácido fórmico al 0.1% (v/v) con un caudal de 0.3 mL min^{-1}. El disolvente orgánico aumentó del 5 al 95% en los primeros 20 min y luego al 100% en los 2 min siguientes; luego, la columna se reajustó a las condiciones iniciales y permaneció en este estado durante 10 min para permitir el reequilibrio de la columna antes de la siguiente inyección. 37 min fue el análisis de tiempo cromatográfico total. Este modo permitió la detección de 17 productos farmacéuticos (codeína, propifenazona, atenolol, carbamazepina, claritromicina, diazepam, fenofibrato, lorazepam, metoprolol, ofloxacino, paroxetina, propanolol, sotalol, sulfadiazina, sulfametazina, sulfametoxazol, y trimetoprima), incluidos los compuestos marcados isotópicamente. Para el modo de ionización negativa (NI), se aplicó un gradiente de ACN: MeOH (1: 1, v/v)/H2O con un caudal de 0.2 mL min^{-1}. En este caso, se programó que el solvente orgánico aumentara del 20 al 80% en los primeros 15 minutos y luego al 90% en los 2 minutos siguientes; posteriormente, la columna se reajustó a las condiciones iniciales programando la cantidad de disolvente orgánico al 20% en 3 min. Estas condiciones permanecieron durante 10 min para permitir el reequilibrio de la columna antes de la siguiente inyección. El tiempo total de análisis cromatográfico fue de 30 min. En este modo, se analizaron 10 compuestos (ketoprofeno, naproxeno, diclofenaco, indometacina, ibuprofen y acetaminofén, bezafibrato, furosemida, gemfibrozil and hidroclorotiazida), incluidos los compuestos marcados isotópicamente. En ambos modos, el volumen de inyección fue de 20 μL. Para el análisis cuantitativo, se realizaron los métodos ESI-MS/MS, según lo descrito por Gros et al. (2009). Las transiciones del ion precursor a dos de sus iones de fragmentos principales se registraron mediante el seguimiento de la reacción seleccionada (SRM) para cada compuesto diana. Se seleccionó una transición SRM para cada estándar sustituto, ya que se agregaron en una concentración lo suficientemente alta como para ser cuantificado de manera confiable en su transición más intensa. Las transiciones de SRM seleccionadas para cada analito y compuesto marcado isotópicamente y las condiciones instrumentales óptimas establecidas para su análisis, así como los compuestos diana y los sustitutos se enumeran en las Tables S1 y S2 del Capítulo1 (Chapter 1) para los AIAPs y en las Tables S3 y S4 del Capítulo 2

(Chapter 2) para los demás compuestos farmacéuticos. Cualquier información adicional se puede consultar en López-Serna et al. (2010).

5.4. Espectrometría de masas (MS) y condiciones MS/MS para el análisis en muestras de fangos

Se utilizó el sistema SCIEX X500R QTOF (Sciex, Redwood City, CA, EE. UU) con una fuente Turbo V™ e ionización por electro-pulverización (ESI) y operando en polaridad negativa/positiva para la detección de los compuestos de interés. Cualquier variación en la precisión de masa del SCIEX Q-TOF se corrigió automáticamente y se mantuvo durante la adquisición del lote mediante la infusión del estándar de referencia de reserpina ($C_{33}H_{40}N_2O_9$, m/z 609.28066) para ionización positiva, y un grupo de ácido trifluoroacético (5(TFA-Na)+TFA-, m/z 792.85963) para modo negativo. La calibración se realizó cada 5 muestras durante la adquisición del lote haciendo uso del Calibrant Delivery System (CDS).

Los datos de alta resolución se adquirieron utilizando un flujo de trabajo de monitoreo de reacciones múltiples (MRMHR) que consta de un levantamiento TOF-MS (100-850 Da para 80 ms de tiempo de acumulación (AT); el potencial de des-agrupamiento (DP) y la energía de colisión (CE) se establecieron en 80 V y 10 V y -80 V y -10 V, para positivo y negativo, respectivamente); el modo de escaneo MRMHR se utilizó para la cuantificación precisa de las transiciones de iones de productos. La herramienta Guided MRMHR de SCIEX se utilizó para la optimización de transiciones de alta resolución (Tabla S1 y S2 del Capítulo 3). El modo de ionización optimizado, los voltajes de fragmentación y las energías de colisión para cada compuesto se han proporcionado como material complementario (Tabla S1, S2; Figura S3, S4, S5 y S6 del Capítulo 3). Las condiciones de la fuente para el sistema se optimizaron de la siguiente manera. El voltaje de pulverización de iones se estableció en 5500 V (-4500 V para negativo); la temperatura de la fuente y los flujos de gas nitrógeno (gas atomizador, GS1 y gas auxiliar, GS2) se establecieron en 550 °C, 50 y 70 psi, respectivamente. El gas de cortina se fijó en 35 psi.

Se realizaron análisis cualitativos y cuantitativos utilizando el software SCIEX OS™ versión 1.4 (Sciex, Redwood City, CA, EE. UU.).

6. Extracción de ADN, qPCR, secuenciación paralela masiva y análisis de datos para el análisis cuantitativo y cualitativo de la comunidad microbiana

6.1. Extracción de ADN de las muestras de fango activo y de digestión

Para las extracciones de ADN, se centrifugaron las muestras biológicas (4 ml) procedentes de fango activo y de fangos sometidos a digestión (1 min, 14.000 × g), que fueron procesadas inmediatamente para su análisis. El ADN se extrajo de las muestras de fango activo utilizando el kit SPIN FastDNA-2 mL para suelo (MP-Bio, Santa Ana, CA, EE. UU.) y el dispositivo FastPrep24, siguiendo los protocolos descritos anteriormente (Maza-Márquez et al., 2016a). La calidad y cantidad de ADN genómico se midió espectrofotométricamente y se calcularon las relaciones A260/A280 usando un espectrofotómetro NanoDrop ND-100 (Thermo Scientific Waltham, MA, EE. UU.).

6.2. Análisis qPCR para muestras de fango activo y digerido

El número de copias de genes marcadores de bacterias totales, arqueas, Mycolata y hongos en las muestras de fango activo y fango digerido se cuantificaron mediante qPCR, utilizando un sistema de qPCR Mx3000P (Agilent Technologies). Las amplificaciones de qPCR se optimizaron utilizando la ADN polimerasa TrueStart Hot Start (Thermo Scientific, Waltham, MA EE. UU.) y SYBR Green I (Sigma Aldrich, St. Louis, MO, EE. UU.). Los cebadores y las condiciones de los ciclos se resumen en la Tabla S3 del Capítulo 1. Cada reacción de qPCR contenía 0,125 μL de ADN polimerasa Taq (50 U/μL), 0,5 μL de cada cebador (10 μM), 2,5 μL de tampón Taq 10×(con KCl), 1,5 μL de $MgCl_2$ (25 mM), 0,5 μL de NTPs (10 mM), 0,0625 μL de albúmina de suero bovino (BSA, 20 mg/mL) y 0,125 μL de SYBR verde diluido en DMSO (20×). El volumen final de las reacciones fue de 25 μL. Todas las amplificaciones cuantitativas se realizaron por triplicado.

Las cuantificaciones absolutas se lograron construyendo curvas estándar utilizando una serie de diluciones con una diferencia de diez veces (10^{-1}-10^{-8}) de plásmidos linealizados que albergan insertos amplificados por PCR de los genes marcadores diana. Los amplicones se generaron a partir de *Pseudomonas putida* NCB 957 (cuantificación de bacterias totales), *Mycobacterium phlei* DSM 43239 (cuantificación de Mycolata), *Methanothrix soehngenii* DSM 3671 (cuantificación de arqueas total) y *Candida albicans* cepa ATCC 10231 (cuantificación de hongos). Los productos de PCR se clonaron utilizando el sistema TOPO® TA cloning® (Invitrogen, Life Technologies Corporation, Carlsbad, CA, EE. UU.), según los protocolos del fabricante. La eficacia de la amplificación por PCR para todos los genes marcadores diana estuvo entre el 90 y el 100%. Las curvas de calibración mostraron un coeficiente de correlación r^2>0.99 en todos los casos. Los rangos de los valores de Ct de las muestras de lodos activados siempre se incluyeron en el rango de detección de los valores de Ct de las curvas estándar. La calidad

de toda la amplificación de qPCR se verificó mediante electroforesis en agarosa y mediante análisis de la curva de fusión. El número de copias de los genes marcadores seleccionados se expresó por L de muestra de fango activo o digerido.

6.3. Illumina Miseq sequencing y procesamiento de datos

Los conjuntos de ADN genómico extraídos se secuenciaron en las instalaciones de RTL Genomics (Lubbock, Texas, EE. UU., Http://www.researchandtesting.com), utilizando la plataforma Illumina MiSeq (Illumina, Hayward, CA, EE. UU.). Los pares de cebadores 28F-519R (5´-GAGTTTGATCNTGGCTCAG-3´ y 5´-GTNTTACNGCGGCKGCTG-3´) (Fan et al., 2012), 517F/909R (5´-GCY-TAAAGSRNCCGTAGC-3´ y 5´-CCCCGYCAATTCMT-3`) (Maspolim et al., 2015b) y FungiQuantF/FungiQuantR (5´-GGRAAACTCACCAGGTCCAG-3´ y 5´-GSWCTATCCCCAKCACGA-3´) (Liu et al., 2012) fueron elegidos para la amplificación parcial del gen 16S rRNA de bacterias y arqueas y el gen 18S rRNA de hongos, respectivamente.

Los datos de secuenciación brutos se procesaron utilizando el software QIIME,v. 1.9.1, siguiendo el protocolo de pipeline previamente descrito por Caporaso et al. (2012). Las secuencias se sometieron a un filtrado de calidad como describieron previamente Bang-Andreasen et al. (2017), que eliminó lecturas de baja calidad (puntuación Q≤25), lecturas con homopolímeros de más de 6 nt, nucleótidos ambiguos (n≥6) o llamadas de base consecutivas de baja calidad (n≥5). Las secuencias quiméricas se eliminaron con USEARCH (Edgar, 2010). Finalmente, las lecturas de secuencia única que ocurrieron en una sola muestra fueron eliminadas de PHY_table_BIOM para análisis adicionales. Las asignaciones taxonómicas de *referencia cercana* basadas en una identidad de secuencia del 97% se realizaron con QIIME v. 1.9.1 (Caporaso et al., 2010) utilizando la base de datos Greengenes (versión 13_08) para las secuencias de Bacteria y arqueas, mientras que la base de datos ARNr 18S eucariota SILVA (versión 111) se utilizó para hongos (Yarza et al., 2017). Los índices descriptivos de diversidad alfa Chao-1, Shan-non-Wiener H 'y Simpson se calcularon utilizando QIIME. Los índices de cobertura de Good también se calcularon como se describió anteriormente (Good, 1953). Las curvas de muestreo de rarefacción y extrapolación (R/E) basadas en el tamaño de la muestra y en la cobertura con intervalos de confianza del 95% se calcularon y se trazaron utilizando la herramienta gratuita en línea 'iNEXT' (Chao et al., 2016; Hsieh et al., 2016), con el fin de hacer comparaciones justas entre muestras de tamaño desigual. Los mapas de calor que muestran la abundancia relativa

de bacterias, arqueas y hongos en cada muestra se generaron utilizando R v.3.2.0 (http://www.r-project.org). Los comandos SIMPROF y SIMPER del software Primer (PRIMER-E v. 6.1.18, Plymouth, Reino Unido) se utilizaron para construir dendrogramas UPGMA con intervalos de confianza del 95% y para estimar la contribución de PHY individuales a la (dis)similitud entre grupos de muestras, respectivamente, siguiendo los métodos ya descritos por (Maza-Márquez et al., 2016b).

7. Análisis estadísticos

Los análisis de distribución de datos y posterior comparación estadística entre grupos de muestras se realizaron utilizando IBM SPSS Statistics v. 19 (SPSS Inc., IBM, EE. UU.). La prueba de Shapiro-Wilk se realizó para evaluar la normalidad de los conjuntos de datos, con el fin de seleccionar el análisis estadístico más apropiado para buscar diferencias entre los grupos de muestras. Como la mayoría de los conjuntos de datos biológicos no se ajustaban a la distribución normal, se seleccionó la prueba no paramétrica de Kruskal-Wallis, con un nivel de significancia del 95% ($p < 0.05$). Cuando fue necesario, se calcularon los coeficientes de correlación de rangos de Spearman (r) entre las diferentes variables.

Todos los análisis estadísticos multivariantes se realizaron utilizando el software Primer (PRIMER-E v. 6.1.18, Plymouth, Reino Unido). Se utilizó la escala multidimensional no métrica (MDS) para: 1) la eficiencia de remoción (RE, expresada como %) de los AIAPs en línea de aguas entre Fase I y II; 2) las abundancias absolutas de los grupos microbianos objetivo cuantificados por qPCR en las muestras de fango activo; y 3) para la ordenación de muestras de fangos activos y digeridos en función de los conjuntos de datos bióticos (abundancias relativas de PHY de los grupos objetivo, derivadas de la plataforma de secuenciación de Illumina) siguiendo los procedimientos descritos anteriormente (Maza-Márquez et al., 2016a). También se realizó un análisis de similitud (ANOSIM). La ordenación de las muestras se vinculó con las tendencias seguidas por las variables abióticas (RE de los PhAC objetivo y variables ambientales/operativas que influyen en el sistema A^2O o en sistema de digestión bifásica) mediante análisis BIO-ENV. Los detalles completos son descritos por Maza-Márquez et al. (2016b).

BIO-ENV calcula las correlaciones de rango de Spearman (ρs) entre dos matrices de (dis) similitud: la matriz fija de Bray-Curtis basada en los datos bióticos medidos en el conjunto de muestras, y las matrices basadas en la distancia euclidiana derivadas de todas las posibles combinaciones secuenciales de las variables abióticas. El valor más alto de ρs

obtenido se denomina MEJOR valor, que designa qué subconjunto de las variables abióticas coincide mejor (= explica) las similitudes de los datos bióticos entre muestras. La significancia estadística de los MEJORES valores se probó mediante una prueba de permutación global (499 permutaciones). Las relaciones entre la ordenación de muestras y las variables abióticas se representaron mediante vectores en los gráficos MDS, que ilustran posibles correlaciones monótonas entre ellas. Primer generó dichos vectores mediante un algoritmo de correlación parcial múltiple, utilizando el coeficiente de correlación de rango de Spearman. En las parcelas, la dirección y longitud de cada vector muestran el signo de la relación y la fuerza de la correlación entre la ordenación de muestras y las tendencias de las variables abióticas (Maza-Márquez et al., 2016a). Finalmente, se calcularon los coeficientes de correlación del momento del producto de Pearson (r) entre los vectores.

IV. RESULTS

❖ CHAPTER 1

Removal of anti-inflammatory/analgesic drugs from urban wastewater in a pilot-scale A^2O system: linking performance and microbial population dynamics to operating variables

Este capítulo ha sido publicado en "Science of the Total Environment". La referencia completa es la siguiente:

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Abstract

In this study, the removal rates of eight anti-inflammatory and/or analgesic pharmaceuticals, AIAPs (acetaminophen, ibuprofen, naproxen, ketoprofen, diclofenac, codeine, indomethacin and propyphenazone) were assessed in a pilot-scale A^2O system (including anaerobic/anoxic/aerobic zones), long term operated during two experimental phases using different sets of environmental conditions and operating parameters. qPCR was used to quantify the absolute abundances of total Bacteria, total Archaea, mycolic-acid containing filamentous Actinobacteria (Mycolata) and Fungi within the activated sludge microbial community developed in the system. Multivariate analyses and Spearman correlation coefficients were used in search of significant links among the removal rates of the AIAPs, the abundances of the targeted microbial groups in the activated sludge, and the changes of environmental/operating variables in the A^2O system. Improved removal efficiencies of several of the AIAPs analyzed (acetaminophen, ibuprofen, naproxen, ketoprofen) were correlated to higher organic load in the influent water, higher concentration of mixed liquor suspended solids (MLSS), lower temperature and lower food-to-microorganisms ratio (F/M). Removal efficiencies of several pharmaceuticals correlated with increased abundances of Mycolata in the A^2O system, pointing at this group of bacteria as candidate key players for AIAPs removal in activated sludge.

1. Introduction

In recent decades, the presence of Pharmaceutically Active Compounds (PhACs) in different environmental compartments (agricultural soils, surface and groundwater systems) has caused great concern among the population. In this regard and echoing this situation, the European Union raised for first time the environmental risks of PhACs (EU, 2013, Directive39/2013) and later included several PhACs such as diclofenac, steroid hormones and macrolide antibiotics erythromycin, clarithromycin and azithromycin in a watchlist of priority substances (EU, 2015, Decision 2015/495).

Among PhACs, anti-inflammatory and/or analgesic pharmaceuticals (AIAPs), either monocyclic (for example ibuprofen, acetaminophen or salicylic acid) or polycyclic (diclofenac, ketoprofen, indomethacin or naproxen) have been extensively detected in water bodies (Kasprzyk-Hordern et al., 2009; Luo et al., 2014; Petrie et al., 2015; He et al., 2017). The source of AIAPs contribution to the environment is the anthropogenic activity that mostly includes human excretion in their parent forms or as metabolites, wastewater disposal from manufacturing lines, or direct dumping of expired products (Wu et al., 2012; Dahane et al., 2013). These compounds reach urban wastewaters and consequently wastewater treatment plants (WWTPs), where they are usually detected at levels ranging from ng L^{-1} to μg L^{-1}, being ibuprofen and acetaminophen, the pharmaceuticals measured at higher concentrations (Radjenović et al., 2009; Verlicchi et al., 2012; Almeida et al., 2013). There, AIAPs undergo several different biological or physicochemical processes, depending of the target compound characteristics and the operating parameters of the WWTP (Clara et al., 2005; Grandclément et al., 2017). The removal efficiencies (RE) of these PhACs in WWTPs are highly variable, being ibuprofen or acetaminophen those degraded at higher rates and diclofenac the most recalcitrant molecule (Joss et al., 2005). In addition, a large number of AIAPs are currently not subjected to the water quality legislation, partly due to a lack of knowledge of their fate and impact in wastewater and the environment (Petrie et al., 2015).

The presence of some AIAPs in treated wastewater effluents and their routine detection in terrestrial and aquatic ecosystems confirms the insufficiency of the conventional activated sludge (CAS) systems for their complete removal. As an alternative, A^2O systems are advanced wastewater treatment processes based on a single sludge configuration using a combination of anaerobic/anoxic/aerobic bioreactors in series, which allows the simultaneous biological removal of organic matter, N and P compounds

(Tchobanoglous et al., 2003). The sequential combination of bioreactor units providing different aeration conditions supports microbial heterotrophic growth, nitrification, denitrification, and biological accumulation of polyphosphates (Kim et al., 2013). In addition, A^2O systems provide advantages over other biological nutrient removal processes, such as a simpler and stable operation, lower energy requirements and reduced operating cost (Tchobanoglous et al., 2003). The coexistence of different functional groups of microorganisms in WWTPs is assumed to result in increased capabilities for the biological transformation of a wider range of organic pollutants (Radjenović et al., 2008; Lai et al., 2011).

At present, the knowledge of microbial biodegradation/biotransformation of AIAPs in WWTPs is still limited. Microbial degradation of polycyclic AIAPs has been achieved; however, most available studies are focused only on the use of pure culture inoculants of fungi and bacteria and are often developed under axenic conditions (Chen and Rosazza, 1994; Domaradzka et al., 2015; Marchlewicz et al., 2016; Grandclément et al., 2017). In contrast, the links among AIAPs removal performance and the population dynamics of the global activated sludge community remain virtually unexplored. In this study, the removal rates of eight AIAPs (acetaminophen, ibuprofen, naproxen, ketoprofen, diclofenac, codeine, indomethacin and propyphenazone) were assessed in a pilot-scale A^2O system, long term operated during two experimental phases using different sets of environmental conditions and operating parameters. qPCR was used to quantify the absolute abundances of total Bacteria, total Archaea, mycolic-acid containing filamentous Actinobacteria (Mycolata) and Fungi within the activated sludge microbial community developed in the system. Multivariate analyses and Spearman correlations coefficients were used in search of significant links among the removal rates of the AIAPs, the abundances of the targeted microbial groups in the activated sludge, and the changes of environmental/operating variables in the A^2O system.

2. Materials and methods

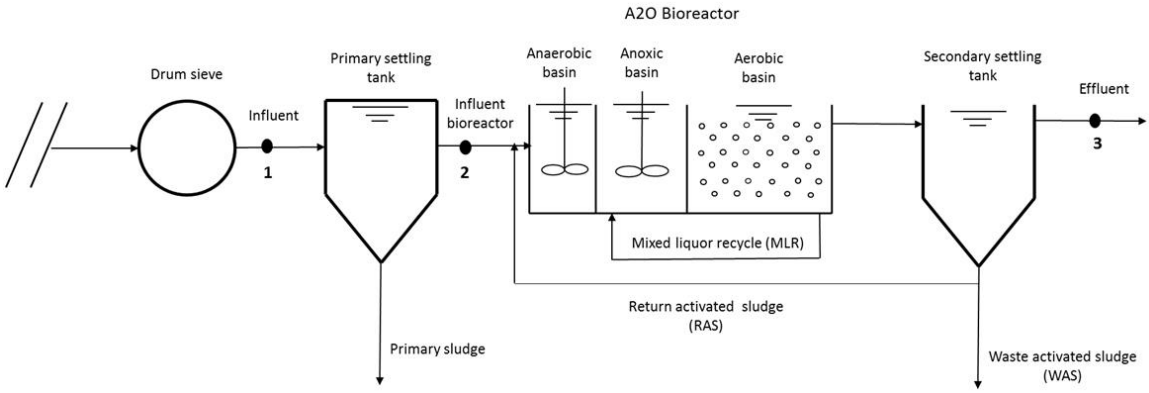
2.1. Description of the wastewater treatment pilot-scale plant and operational conditions

In this investigation, a pilot-scale A^2O plant located at the facilities of the WWTP Murcia Este (EMUASA, Murcia, Spain) was used. The Murcia Este plant was designed for 833,000 population equivalents with an average flow rate of 100,000 m^3 day^{-1}. The pilot

plant (Figure 1) is a scale-copy (1:25000) of the WWTP Murcia Este, designed to treat up to 6 m^3 per day, and consisted of a 0.512 m^3 primary sedimentation tank, a 1.66 m^3 bioreactor tank (anaerobic basin: 0.173 m^3, anoxic basin: 0.277 m^3, aerobic basin:1.200 m^3) and a 0.616 m^3 clarifier. The pilot plant was fed with wastewater taken from the pre-treatment unit of the full-scale WWTP Murcia Este, and it was a mixture of municipal and industrial wastewater.

The pilot-scale plant started up on May, 20th 2016, with activated sludge from the WWTP Murcia Este as inoculum. Since June 1st, the plant was operated with the working concentration of mixed liquor suspended solids (MLSS) (2500–3000 mg L^{-1}) and food-to-microorganism ratios (F/M) (over 0.30 kgBOD_5 kg$MLVSS^{-1}$ d^{-1}) selected for phase I. Steady-state conditions were reached after about threefold the SRT, then the sampling campaign started. After phase I experimentation ended, a transition phase was required for stable operation under the working conditions selected for phase II (4000–5600 mg L^{-1} MLSS and F/M over 0.20 kg BOD_5 kg $MLVSS^{-1}$ d^{-1}). Phase I (July 27th–November 13th 2016) and phase II (December 14th 2016–March 31th 2017) lasted 104 and 105 days, respectively. Although each experimental phase was characterized by different MLSS and F/M, similar hydraulic retention times (HRT) were maintained. The monthly average values for each operational parameter in each sampling period are reported in Table 1.

Figure 1. Schematic diagram of the pilot-scale plant used in the study. Sampling points 1, 2 (influent) and 3 (effluent) are indicated.



2.2. Wastewater sampling collection for the analysis of physicochemical parameters and AIAPs

Daily-composite samples were taken three times per week from sampling points 1, 2 and 3 of the A^2O system (Figure 1) to determine the physicochemical parameters described

in Section 2.3. A total of eighteen influent and effluent wastewater samples were collected for AIAPs analysis: eight samples in phase I (four in Summer and four in Autumn) and ten samples in phase II (two in Autumn, four in Winter, and four in Spring). 24-h composite samples were taken from sampling points 1 and 3 of the pilot scale plant (Figure 1). Samplings were done both mid-week and at the end of the weekend. The samples were collected in 500-mL amber PET bottles and frozen at −20 °C until their analysis. The effluent samples were taken according to the constant HRT (12 h). All samples were collected in dry weather conditions to eliminate sample dilution effect.

2.3. Analysis of physicochemical parameters in the A^2O system

Merck Spectroquant® kits (Darmstadt, Germany) were used to measure chemical oxygen demand (COD) (100 - 1500 mg L^{-1}, 10 - 150 mg L^{-1}), total nitrogen (TN) (10 - 150 mg L^{-1}), N-NO_3^- (1.0-50.0 mgL^{-1}), N-NH_4^+ (4.0 - 80.0 mg L^{-1}, 0.010 - 2.000 mg L^{-1}), and total phosphorous (TP) (0.5 – 25 mg L^{-1}). Biological oxygen demand (BOD_5), total suspended solids (TSS), MLSS and mixed liquor volatile solids suspended (MLVSS) were determinate according to *Standard Methods for the Examination of Water and Wastewater* (Baird et al., 2017).

2.4. Analytical methods for AIAPs

2.4.1. Chemicals

All chemical standards used for the analyses were purchased from Sigma-Aldrich (St Louis, MO, USA) and Cerilliant (Round Rock, TX, USA). Isotopically labeled compounds employed for internal standard calibration were purchased from Sigma-Aldrich (St Louis, MO, USA) and Santa Cruz Biotechnology (Dallas, TX, USA). All standards were of purity grade (>90 %) (listed in Supplementary Material). Individual stock solutions of the standards (100 mg L^{-1} in MeOH) were prepared. A mix of compounds (10 µg mL^{-1} in MeOH) was prepared every three months for calibration and spikes by appropriate dilution in H_2O/MeOH 95:5 v/v. A mix of isotopically labeled compounds was prepared separately under the same conditions explained before. All stock solutions were stored in the dark at -20°C. HPLC grade solvents were purchased from Merck (Darmstadt, Germany): MeOH (purity 99.9% or greater), ACN (purity 99.9% or greater), and water.

2.4.2. Sample pretreatment

Sample pretreatment was performed following previously described methods (Gros et al., 2008; López-Serna et al., 2010). Briefly, water samples were filtered through 1µm fiberglass filters (Whatman, Fairfield, Connecticut, USA), followed by filtration through 0.45µm nylon membrane filters (Teknokroma, Barcelona, Spain). Finally, 10 µL of a 100 ng mL^{-1} mixture of internal standards was spiked in a 10 mL of sample for surrogate control and internal standard calibration.

2.4.3. On-line extraction

Pre-concentration of wastewater samples and chromatographic separation was performed in an automated on-line SPE–LC device Symbiosis™ Pico (Spark Holland, Emmen, The Netherlands) in online SPE mode using SPE cartridges HySphere Resin GP 10 (polydivinyl-benzene, 5–15-µm particle size) (Spark Holland, Emmen, The Netherlands). The cartridge was conditioned with 2 mL MeOH and 2 mL water, at a flow rate of 5 mL min^{-1}, then 2.5 mL of sample was loaded into the cartridge at a flow rate of 1 mL min^{-1} to improve analytes retention onto the sorbent. Before elution, the cartridge was rinsed with 1 mL HPLC grade water at a flow rate of 5 mL min^{-1} to complete the transfer of the sample and remove interferences. Finally, analytes were eluted by the "standard" approach, where the full chromatographic gradient passes through the SPE cartridge before being led into the LC column. During elution, a new cartridge was placed in pre-concentration mode for the next sample. This way, elution of one sample and pre-concentration of a second sample were carried out simultaneously. All gradient eluted passed through the LC column for a chromatographic separation.

2.4.4. HPLC separation and ESI-QqLIT MS/MS analysis

The gradient eluted passed through a reversed-phase Purospher Star RP-18 end-capped column (125mm×2.0mm, particle size 5µm) (Merck, Darmstadt, Germany) for the separation of compounds. For mass spectrometry detection, Symbiosis™ Pico operated in extraction LC mode connected in series with a 4000QTRAP hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer, equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, California, USA). 4000QTrap is controlled by Analyst 1.4.2 Software (Applied Biosystems-Sciex, Foster City, California, USA) and a company software appendix for controlling the Symbiosis™ Pico (Spark Holland, Emmen, The Netherlands).

Elution of analytes was performed following previously described methods (Gros et al., 2008). Briefly, for the positive ionization (PI) mode, a gradient of ACN/formic acid 0.1% (v/v) with a flow rate of 0.3 mL min^{-1} was applied. Organic solvent increased from 5 to 95% in the first 20 min and then to 100% in the following 2min; then, the column was readjusted to the initial conditions and remained in this state for 10 min to allow re-equilibration of the column before the next injection. 37 minutes was the total chromatographic time analysis. This mode allowed detection of two pharmaceuticals (codeine and propyphenazone) including isotopically labeled compounds. For the negative ionization (NI) mode, a gradient of ACN:MeOH (1:1, v/v)/H_2O with a flow rate of 0.2 mL min^{-1} was applied. In this case, organic solvent was programmed to increase from 20 to 80% in the first 15 min and then to 90% in the following 2 min; subsequently, the column was readjusted to the initial conditions by programming the amount of organic solvent to 20% in 3 min. These conditions remained for 10 min to allow re-equilibration of the column before the next injection. The total time of chromatographic analysis was 30 min. In this mode, six compounds (ketoprofen, naproxen, diclofenac, indomethacin, ibuprofen and acetaminophen) were analyzed including isotopically labeled compounds. In both modes, the injection volume was 20 µL. For quantitative analysis, the ESI-MS/MS methods were performed, as described by Gros et al. (2008). Transitions from the precursor ion to two of its main fragment ions were registered by selected reaction monitoring (SRM) for each target compound. One SRM transition was selected for each surrogate standard, since they were added in high enough concentration to be reliably quantified in its more intense transition. Selected SRM transitions for each analyte and isotopically labeled compound and the optimal instrumental conditions set for their analysis are summarized in Table S1. Target compounds and surrogates are listed in Table S2. Any additional information can be consulted in López-Serna et al. (2010).

2.4.5. Activated sludge samplings for qPCR

200 mL of activated sludge from the aerobic compartment of the A^2O bioreactor were sampled in each experimental phase when the steady-state conditions were reached (at least threefold the sludge retention time, SRT, since the start-up date). For DNA extractions, three biological samples (4 mL) of activated sludge were centrifuged (1 minute, 14×g) and immediately processed for analyses. DNA was extracted from the activated sludge samples using the FastDNA-2 mL SPIN kit for Soil (MP-Bio, Santa Ana, CA, USA) and the FastPrep24 apparatus, following previously described protocols (Maza-Márquez et al.,

2016). The quality and quantity of genomic DNA was measured spectrophotometrically and A260/A280 ratios were calculated using a NanoDrop ND-100 Spectrophotometer (Thermo Scientific Waltham, MA USA).

2.5. qPCR assays

The numbers of copies of marker genes of total Bacteria, total Archaea, Mycolata and Fungi in the activated sludge samples were quantified by qPCR, using an Mx3000P qPCR system (Agilent Technologies). qPCR amplifications were optimized using TrueStart Hot Start DNA polymerase (Thermo Scientific, Waltham, MA USA) and SYBR Green I (Sigma Aldrich, St. Louis, MO, USA). Primers and cycling conditions are summarized in Table S3. Each qPCR reaction contained 0.125 µl of Taq DNA polymerase (50 U/µl), 0.5µl of each primer (10 µM), 2.5 µl of 10×Taq Buffer (with KCl), 1.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM), 0.0625 µl of bovine serum albumin (BSA, 20 mg/ml), and 0.125 µl of SYBR green diluted in DMSO (20×). The final volume of the reactions was 25 µl.

Absolute quantifications were achieved by constructing standard curves using serial tenfold dilutions (10^{-1}-10^{-8}) of linearized plasmids harboring PCR-amplified inserts of the targeted marker genes. Amplicons were generated from *Pseudomonas putida* NCB 957 (quantification of total Bacteria), *Mycobacterium phlei* DSM 43239 (quantification of Mycolata), *Methanothrix soehngenii* DSM 3671 (quantification of total Archaea) and *Candida albicans* strain ATCC 10231 (quantification of fungi). PCR products were cloned using the TOPO® TA cloning® system (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA), according the manufacturer's protocols. The efficiency of PCR amplification for all targeted marker genes was between 90 and 100%. The calibration curves showed a correlation coefficient $r^2 > 0.99$ in all the assays. The ranges of the Ct values of the activated sludge samples were always included in the range of the detection of Ct values of the standard curves. The quality of all qPCR amplification was verified by electrophoresis in agarose and by melting curve analysis. The numbers of copies of the selected marker genes were expressed per L of activated sludge samples.

2.6. Statistical analysis

Analysis of data distribution and subsequent statistical comparison between groups of samples were performed using IBM SPSS Statistics v. 19 (SPSS Inc., IBM, USA). The Shapiro-Wilk test was conducted to assess the normality of the data sets, in order to select

the most appropriate statistical analysis to search for differences among the groups of samples. As most of the biological data sets did not fit the normal distribution, the Kruskal-Wallis non-parametric test was selected, with a 95% significance level ($p < 0.05$).

The Primer software (PRIMER-E v. 6.0, Plymouth, UK) was used for the analysis of: 1) the removal efficiency (RE, expressed as %) of the AIAPs in the A^2O system, and 2) the absolute abundances of the targeted microbial groups quantified by qPCR in the activated sludge samples, following the procedures described elsewhere (Maza-Márquez et al., 2016). Briefly, both data sets were standardized by total, and sample-resemblance matrices were generated using the Bray Curtis coefficient of similarity. Based on the similarity matrices, Non-metric Multidimensional Scaling (MDS) was performed to display the samples' ordination in plots, either based on the REs of AIAPs or the abundances of the targeted microbial groups. The stress level of the MDS plots was <0.2, indicating that the biotic data distribution were validly represented in 2D-plots (Clarke and Warwick, 2001).

The operational parameters and environmental variables influencing the A^2O system (herein abiotic variables) were linked to the REs of AIAPs and the biological patterns derived from qPCR by means of BIO-ENV analysis and the calculation of BEST values, using Primer (Maza-Márquez et al., 2016). BIO-ENV computes the Spearman rank correlations (ρs) between two (dis)similarity matrices: the Bray-Curtis fixed matrix based on the biotic data measured in the set of samples, and the Euclidean-distance based matrices derived from all the possible sequential combinations of the abiotic variables. The highest value of ρs obtained is called the BEST value, which designates which subset of the abiotic variables best matches (=explains) the similarities of the biotic data between samples. The statistical significance of the BEST values was tested by a global permutation test (499 permutations). With the exception of pH, the abiotic data sets were transformed to log (x+1) and normalized. Relationships between the ordination of samples and the abiotic variables were represented by vectors in the MDS plots, which illustrate potential monotonic correlations between them. Primer generated such vectors by a multiple partial algorithm of correlation, using the Spearman rank correlation coefficient. In the plots, the direction and length of each vector show the sign of the relationship and the strength of the correlation between the ordination of samples and the trends of the abiotic variables (Maza-Márquez et al., 2016). Finally, the Pearson's product moment correlation coefficients (r) among the vectors were calculated.

3. Results and discussion

3.1. Performance of the pilot-scale plant: A^{2}O bioreactor system

Table 1 shows the monthly average values for each operational parameter in each sampling period and the transition phase among them. The HRT, MLSS, F/M and % of return activated sludge (RAS) values were always kept constant during each phase, whereas the mixed liquor recycle ratio (MLR) was adapted to maintain the N-NO_3^- value below the limit value discharge (LVD) of WWTP Murcia Este. On the other hand, the SRT changed during phase I (between 9.0 and 15.1 days) due to the progressive increase of the organic loading rate (OLR) experienced (Table 1), while it remained constant in phase II (ca. 12 days), except in the first 15 days of phase II (15–31 December 2016) where the SRT increased to 19.8 days, due to a reduction of the waste activated sludge flow (WAS) applied in order to maintain the concentration of MLSS throughout a long period of heavy rainfall.

The main differences in the operational parameters between phases were the concentration of MLSS (2663 ± 434 and 5079 ± 529 mgL^{-1} in phases I and II, respectively), F/M (0.34 and 0.21 kg BOD_5 $MLVSS^{-1}$ d^{-1} in phases I and II, respectively) and OLR (0.625 ± 0.173 and 0.851 ± 0.272 kg BOD_5 m^{-3} day^{-1} in phases I and II, respectively). On the contrary, the average values of HRT, SRT and nitrogen loading rate (NLR) remained similar in both phases.

Table 2 shows the mean concentrations of COD, BOD5, TSS, TN, N-NH_4^+, N-NO_3^-, TP, turbidity and pH in the bioreactor's influent (point 2, Figure 1) and effluent (point 3, Figure 1) wastewater samples during phases I and II in the A^{2}O system

Table 1. Monthly average values and global average values (± standard deviation) for operational parameters of the A²
II (B.). Data marked with an asterisk (*) are significantly different in phases I and II, according to the Kruskal-Wallis te

A.			**Phase I**		
Parameter	**27-31 July**	**August**	**September**	**October**	**01-**
Influent flow (L h^{-1})	237 ± 22	255 ± 24	230 ± 24	230 ± 22	230
HRT (h)	7.0 ± 0.7	6.5 ± 0.6	7.2 ± 0.7	7.2 ± 0.7	7.2
RAS (%)	54 ± 5	58 ± 5	57 ± 6	61 ± 6	52 ±
MLR (%)	96 ± 9	128 ± 12	158 ± 16	214 ± 20	211
SRT (d)	15.1 ± 2.6	16.9 ± 3.6	11.3 ± 0.8	9.0 ± 1.7	11.4
F/M (kg BOD_5 kg MLVSS $^{-1}$ d^{-1})	0.32 ± 0.02	0.35 ± 0.21	0.44 ± 0.03	0.27 ± 0.03	0.32
SVI (mL g^{-1})	67 ± 5	68 ± 9	74 ± 5	91 ± 10	126
DO set point (mg L^{-1})	1.0 - 2.0	0.5 - 2.0	0.5 - 2.0	0.5 - 2.0	0.5
MLSS (mg L^{-1})	2625 ± 188	2564 ± 575	2734± 360	2626 ± 379	284
MLVSS (%)	77.9 ± 2.8	76.7 ± 2.1	76.8 ± 4.4	80.6± 3.7	82.0
OT (ºC)	25.5 ± 1.7	27.4 ± 1.3	24.9 ± 1.3	21.6 ± 1.6	20.4
OLR (kg BOD_5 m^{-3} d^{-1})	0.725 ± 0.130	0.515 ± 0.150	0.791 ± 0.180	0.639 ± 0.089	0.75
ORR (%)	93.8 ± 0.1	91.7 ± 4.1	93.9 ± 3.5	92.0 ± 3.6	87.5
NLR (Kg TN m^{-3} d^{-1})	0.108 ± 0.010	0.165 ± 0.010	0.198 ± 0.010	0.199 ± 0.010	0.18
NRR (%)	46.9 ± 6.4	49.5 ± 10.4	56.0 ± 6.1	66.2 ± 5.2	62.8
PLR (Kg TP m^{-3} d^{-1})	0.014 ± 0.002	0.015 ± 0.001	0.020 ± 0.001	0.015 ± 0.001	0.01
PRR (%)	60.0 ± 9.4	51.5 ± 15.4	61.1± 11.3	42.3±11.1	41.7

B.	Transition phase				Phas
Parameter	**14-30 Nov**	**01-13 Dec**	**14-31 Dec**	**January**	**Februar**
Influent flow (L h^{-1})	221 ± 23	243 ± 34	249 ± 34	242 ± 28	240 ± 28
HRT (h)	7.5 ± 0.7	6.8 ± 1.0	6.7 ± 0.9	6.9 ± 0.8	6.9 ± 0.8
RAS (%)	78 ± 8	80 ± 11	71 ± 10	67 ± 8	79 ± 9
MLR (%)	197 ± 19	171 ± 24	172 ± 24	196 ± 23	209 ± 23
SRT (d)	12.9 ± 1.2	11.8 ± 1.1	19.8 ± 6.6	12.1 ± 1.9	12.6 ± 1.
F/M (kg BOD_5 kg $MLVSS^{-1}$ d^{-1})	0.25 ± 0.04	0.26 ± 0.23	0.18 ± 0.08	0.21 ± 0.07	0.21 ± 0.
SVI (mL g^{-1})	163 ± 12	157 ± 3	137 ± 19	106 ± 16	107 ± 12
OD (mg L^{-1})	1.0 - 2.0	1.0 - 2.0	0.5 - 2.0	0.5 - 2.0	0.5 - 2.0
MLSS (mg L^{-1})	3968 ± 388	4693 ± 248	4785 ± 342	5127 ± 529	5494 ± 5
MLVSS (%)	82.3 ± 2.6	75.3 ± 8.0	75.4 ± 2.7	78.4 ± 1.8	80.3 ± 2.
OT (ºC)	18.7 ± 1.5	19.4 ± 0.6	16.9 ± 1.6	16.3 ± 2.5	16.2 ± 2.
OLR (kg BOD_5 m^{-3} d^{-1})	0.807 ± 0.085	0.775 ± 0.507	0.677 ± 0.324	0.867 ± 0.301	0.927 ± 0
ORR (%)	93.0 ± 3.3	94.8 ± 1.1	95.5 ± 2.0	95.1 ± 2.0	96.5 ± 1.
NLR (kg TN m^{-3} d^{-1})	0.219 ± 0.038	0.166 ± 0.02	0.146 ± 0.048	0.191 ± 0.011	0.201 ± 0
NRR (%)	73 ± 4.2	72.3 ± 1.1	66.4 ± 6.4	72.4 ± 1.2	66.8 ± 2.
PLR (kg TP m^{-3} d^{-1})	0.022 ± 0.002	0.018 ± 0.006	0.012 ± 0.008	0.018 ± 0.001	0.019 ± 0
PRR (%)	51.5 ± 6	66.7 ± 2.4	70.0 ± 2.3	60.2 ± 1.5	54.4 ± 4.

HRT: hydraulic retention time; RAS: return activate sludge rate; MLR: mixed liquour recycle rate; SRT: sludge retenti ratio; SVI: sludge volumetric index; DO: dissolved oxygen; MLSS: mixed liquor suspended solids; MLVSS: mixed liq operating temperature; Organic loading rate (OLR), nitrogen loading rate (NLR) and phosphorous loading rate (PLR) v Removal Rate (ORR), Nitrogen Removal Rate (NRR) and Phosphorous Removal Rate (PRR). HRT, F/M, RAS, MLR, calculated as described by Tchobanoglous et al. (2003).

Table 2. Average ± standard deviations of physical-chemical parameters measured in influent and effluent water sam
system. Data marked with an asterisk (*) are significantly different in phases I and II, according to the Kruskal-Wallis t

	COD (mg L^{-1})	**BOD_5 (mg L^{-1})**	**TSS (mg L^{-1})**	**TN (mg L^{-1})**	**N-NH_4^+ (mg L^{-1})**	**N-NO_3^- (mg L^{-1})**	**TP (mg**
	Influent						
Phase I	338 ± 57 *	189 ± 43 *	104 ± 30 *	53.4 ± 10.5	40.2 ± 7.5	0.3 ± 0.3	4.8 ± 0.5
Phase II	424 ± 103 *	234 ± 78 *	145 ± 47 *	51.2 ± 10.6	41.5 ± 12.5	0.2 ± 0.4	5.1 ± 0.2
	Effluent						
Phase I	54 ± 14	14 ± 6 *	15 ± 5 *	22.2± 4.8 *	1.6 ± 2.4	10.6 ± 3.7	2.5 ± 0.9
Phase II	52 ± 15	9 ± 4 *	21 ± 7 *	18.3 ± 3.4 *	1.0 ± 3.1	11.1 ± 4.0	1.7 ± 0.7

COD: chemical oxygen demand; BOD_5: biological oxygen demand at 5 days; TSS: total suspended solids; TN

The fluctuations detected in influent samples in both phases corresponded to a typical municipal wastewater (Tchobanoglous et al., 2003). The mean concentration of organic matter and TP in the influent samples of phase I was significantly lower than in phase II, in accordance with the higher operating temperature (OT) values registered in the summer period (24.0 ± 1.3 °C in phase I compared to 17.5± 2.1 °C in phase II), which typically enhance biodegradation rates in the sewer system networks (Tchobanoglous et al., 2003). The mean effluent values were below the LVD of WWTP Murcia Este.

In phase I, the A^2O system achieved 91.6 ± 4.0% organic removal rate (ORR), 56.6 ± 10.7% total N removal rate (NRR), and 49.1 ± 13.2% total P removal rate (PRR), while significantly higher removal rates of organic matter and nutrients were reached in phase II operating at higher MLSS and lower F/M, being the mean values 95.6 ± 2.0%, 67.5 ± 3.9%, and 66.2 ± 13.1% for ORR, NRR and PRR, respectively (Table 1). The improved performance of nutrients removal observed throughout phase II was connected to an increase of the OLR compared to phase I, while NLR was kept in similar values, yielding a higher COD/TN ratio (6.48 in phase I and 7.79 in phase II), as well as the increase of the availability of easily biodegradable organic matter (BOD_5), which influences the ability of polyphosphate accumulating organisms (PAOs) and heterotrophic microorganisms for phosphorous removal and denitrification processes, respectively (Barnard and Abraham, 2006).

The nitrification process was efficiently carried out in both phases with a mean nitrogen ammonium ($N\text{-}NH_4^+$) percentage removal of 96.2% and 96.9% in phases I and II, respectively, even though the dissolved oxygen (DO) on the aerobic zone was maintained at relatively low concentrations (0.5–1.2 mg L^{-1}), compared to the DO value of 2mgL^{-1} recommended in the literature for complete nitrification (Zeng et al., 2010; Leyva-Díaz et al., 2016). Efficient nitrification at low DO values was favored by the long SRT used throughout both experimental phases (average 12.5 days and 13.3 days, phases I and II, respectively), as well as the high OT registered throughout phase I (24 ± 1.3 °C) and the high MLSS concentrations reached during phase II (average 5079 ± 422 mg L^{-1})(Barnard and Abraham, 2006). In addition, simultaneous nitrogen removal via nitrite could occur in the aerobic basin due to the low DO levels (Trivedi, 2009). Operating with the lowest DO possible is desirable in full-scale WWTPs in order to reduce aeration cost and waste sludge (Zeng et al., 2010).

In phase I, the average sludge volumetric index (SVI) was 84 ± 24 mL g^{-1}, indicating an excellent settling of the sludge, and no sludge bulking episodes occurred. In phase II,

the SVI increased over 160 mL g^{-1} during the transition phase and then decreased gradually to 84 mL g^{-1}, with an average value of 106 ± 22 mL g^{-1}. Isolated bulking sludge episodes were observed during the transition phase, and no foaming occurred throughout the whole experimental period. The SVI increment was correlated with the proliferation of filamentous bacteria (Mycolata), as it will be further discussed in Section 3.4

3.2. Occurrence of AIAPs in influent and effluent wastewater samples

Table 3 shows the range, mean, median, relative standard deviation (RSD) and frequency of detection of the targeted AIAPs acetaminophen, ibuprofen, naproxen, ketoprofen, diclofenac, codeine, indomethacin and propyphenazone in the influent and effluent wastewater samples analyzed during experimental phases I and II. All AIAPs influent values fell within the concentration ranges previously reported in wastewater samples by several authors (Verlicchi et al., 2012; Luo et al., 2014).

Similar mean influent concentration values for all the AIAPs analyzed were measured in both experimental phases, except for acetaminophen, which showed a wider range of values (18,200–301,000 ng L^{-1}) and a higher mean value (163,475 ng L^{-1}) in phase I. In both experimental phases, high relative standard deviations (RSD) for acetaminophen average values were calculated in both influent and effluent water, reflecting a large variability of its concentration. These results are in agreement with data of the Spanish Ministry of Health, Social Policy and Equality (2017), which rated acetaminophen as the most consumed pain reliever in Spain in 2015, in both prescription and over-the-counter medicines.

Table S4 shows AIAPs amounts consumed in Spain during year 2016 in terms of DHD (Daily doses defined per 1000 inhabitant and day) and DDD (Daily doses defined, mg active compound day^{-1}). The data (Table 3) were similar to the predicted environmental concentration (PEC) calculated from consumption data of the Spanish Agency of Medicines and Healthcare Products (AEMPS, https://www.aemps.gob.es/medicamentosUsoHumano/observatorio/informes.htm).

In the effluent wastewater samples (Table 3), ibuprofen showed the highest mean value in both phases I and II (8795 ng L^{-1} and 1638 ng L^{-1}, respectively), followed by acetaminophen (6226 ng L^{-1} and 1234 ng L^{-1}), naproxen (4003 ng L^{-1} and 938 ng L^{-1}) and diclofenac (1142 ng L^{-1} and 1266 ng L^{-1}).

Table 3. Concentration range, mean concentration, median concentration, relative standard deviation (RSD) and detec
pharmaceuticals (AIAPs) in the influent and effluent wastewater samples of phases I (n=4) and II (n=5) in the A^2O sy
was used for statistical analyses. The removal of those AIAPs marked with an asterisk (*) was significantly different in
detection.

					Influent (ng L^{-1})		
				Phase I			
AIAPs	**Range**	**Mean**	**Median**	**RSD (%)**	**Freq. (%)**	**Range**	**Mean**
Acetaminophen	18200 – 301000	163475	167350	93	100	2620 - 261000	110942
Ibuprofen	9280 - 25500	15970	14550	43	100	13900 - 24100	20000
Naproxen	4030 - 12300	7762	6950	46	100	4430 - 10800	7264
Diclofenac	1090 - 1720	1293	1180	23	100	777 - 1280	1093
Ketoprofen	893 - 1410	1041	957	24	100	1070 - 1680	1288
Codeine	BLD- 383	192	193	83	75	23.2 - 398	195
Indomethacin	BLD- 64.1	21.2	10.3	138	75	2.19 – 65.2	27.9
Propyphenazone	BLD - 16	4.4	0.8	178	50	2.4-10.5	6.4
					Effluent (ng L^{-1})		
				Phase I			
Analyte	**Range**	**Mean**	**Median**	**RSD (%)**	**Freq. (%)**	**Range**	**Mean**
Acetaminophen	BLD- 21400	6226	1751	164	75	702 - 5410	1234
Ibuprofen *	6420 - 10900	8795	8930	25	100	BLD - 7910	1638
Naproxen *	2790 - 6000	4003	3610	38	100	232 - 2890	938
Diclofenac	967 - 1410	1142	1095	17	100	1090 - 1610	1266
Ketoprofen	590 - 1890	1151	1062	50	100	247 - 1180	737
Codeine	BLD - 319	80	0	200	25	BLD - 352	146
Indomethacin	BLD – 17.6	7.7	6.6	95	75	BLD – 9.4	5.2
Propyphenazone	BLD – 0.7	0.2	0.0	170	25	BLD – 9.7	5.2

The concentrations detected in the effluents were always into the ranges of previously reported data (Verlicchi et al., 2012; Luo et al., 2014), but in phase I they were higher than the mean values detected in phase II for acetaminophen, ibuprofen, naproxen and ketoprofen.

Table 4 shows the daily measured mass load (ML, mg day^{-1}1000 inh^{-1}) for AIAPs in the influent and effluent water samples in each experimental phase. Similar influent ML values were measured in both phases for each target compound. In the effluent samples, ML values were three times lower for naproxen and six times lower for acetaminophen and ibuprofen in the effluent water of phase II, compared with phase I, while similar ML values were found for the rest of the target AIAPs. Those differences found in the effluent are due to the increase of the REs for naproxen, acetaminophen and ibuprofen, as discussed in Section 3.3

According to the available literature, AIAPs usually account for the highest percentage of the ML of PhACs in urban WWTPs over the world. In this sense, Park et al. (2017) reported a total influent mean value of 82% for five WWTP in Korea, 40–70% of which corresponded to acetaminophen. Zorita et al. (2009) reported a 93% in WWTPs in Sweden, while Jelic et al. (2011) obtained a mean value of 65% in three WWTPs in Spain, but acetaminophen and ibuprofen were not considered in their study. Pereira et al. (2015) reported a mean value of 72% for fifteen WWTPs in Portugal, being acetaminophen, the drug contributing the highest ML (16,900 mg day^{-1}1000 inh^{-1}). Overall, the ML data calculated in the present study are slightly higher than those reported by the aforementioned authors, being the data for ibuprofen, naproxen and diclofenac the closest ones to the values observed by Zorita et al. (2009).

Table 4. Daily measured mass load (ML, mg day^{-1} 1000 inh^{-1}) of anti-inflammatory/analgesic pharmaceuticals (AIAPs) detected in the influent and effluent wastewater samples in phases I and II in the A^{2}O system. The predicted daily mass load (PL, mg/day per 1000 inh) for WWTP Murcia Este is included too for comparison.

AIAPs	Phase I		Phase II		PL
	Influent	Effluent	Influent	Effluent	Influent
Acetaminophen	36,819	1402	24,987	278	36,000
Ibuprofen	3597	1981	4505	369	2461
Naproxen	1748	901	1636	211	554
Ketoprofen	234	259	294	166	362
Diclofenac	291	257	246	285	127
Codeine	43	18	44	33	51
Indomethacin	5	2	6	1	30
Propyphenazone	1	0	1	1	30

Total	**42738**	**4820**	**31,719**	**1344**	**39,615**

3.3. AIAPs removal efficiency and links with the environmental/operational variables influencing the A^2O system

Table 5 shows the range, mean, median and relative standard deviation of the REs of the AIAPs throughout the experimental phases I and II in the A^2O system. Acetaminophen was the only drug exhibiting very high RE values in both phases (94 and 99.9%, phases I and II, respectively) and displaying low RSD values, indicating a stable RE regardless of the operational conditions, in agreement with the results of previous studies (Verlicchi et al., 2012; Luo et al., 2014). In contrast, REs of ibuprofen and naproxen were significantly different among the experimental phases (Table 5), with RE in phase I about half the values registered in phase II (39% versus 88% for ibuprofen and 41% versus 87% for naproxen). RDS values were also higher in phase I compared to phase II for both compounds (Table 5). Mean RE of ketoprofen was 0% in phase I, while a 39% mean RE value was obtained in phase II; however, the REs for this compound widely varied and the difference among phases was not significant, according to the Kruskal-Wallis test.

Very low or negative mean REs of diclofenac were observed in phase I (9%) and phase II (−13%), with a maximum RE of 44% and 3% for phases I and II, respectively (Table 5). Accordingly, wide variations in RE for diclofenac have been often reported in municipal WWTPs, ranging from negative REs to values up to 90% (Clara et al., 2005; Kasprzyk-Hordern et al., 2009; Zorita et al., 2009), with the most common REs in the range of 20–50% (Verlicchi et al., 2012).

Wide variability of RE values were obtained for codeine in phase I, ranging from negative values −51% to 100%, with a mean value of 50%. Lower RSD and high detection frequencies were obtained in phase II, with a mean RE value of 42%. These values are below those reported previously (60–70%) in the literature (Kasprzyk-Hordern et al., 2009; Verlicchi et al., 2012). RE mean values for indomethacin were higher in phase I (60%) than in phase II (31%), and they were similar to those previously described. For example, Verlicchi et al. (2012) reported mean RE values of 37% in CAS and 43% in an advanced system based on a membrane bioreactor (MBR), while no removal in CAS and 40% RE in a MBR were observed by Radjenović et al. (2009). Propyphenazone was measured at very low concentrations in the influent wastewater (maximum values 16 ng L^{-1} and 10 ng L^{-1} for phases I and II, respectively) and it was detected only in three samples in phase I, hence

conclusions regarding its RE in this phase could not be properly drawn. In phase II with n=5 pared-samples, the mean propyphenazone RE was 36%, falling within a −15% to 100% range. This average RE is close to those observed in CAS systems by Radjenović et al. (2009) and Verlicchi et al. (2012) (37% and 45%, respectively). The same authors also agreed that removal of propyphenazone from wastewater is significantly improved in MBR systems (60–63% RE).

Several mechanisms have been proposed to explain negative REs of PhACs in WWTPs, as observed for diclofenac in phase II. PhACs can be enclosed in particles, adsorbed on suspended particles, or encapsulated by surfactant molecules, and may be later released when microorganisms break down those particles (Blair et al., 2015). In addition, diclofenac is transformed from the conjugate form to the parent compound by deconjugation of glucuronide or sulfate diclofenac (Verlicchi et al., 2012). Also, compounds occurring at very low concentrations (as in the case of codeine, indomethacin and propyphenazone) may yield negative REs due to analytical limitations. In this sense, punctual negative RE values were also measured for ketoprofen, codeine, indomethacin and propyphenazone (Table 5).

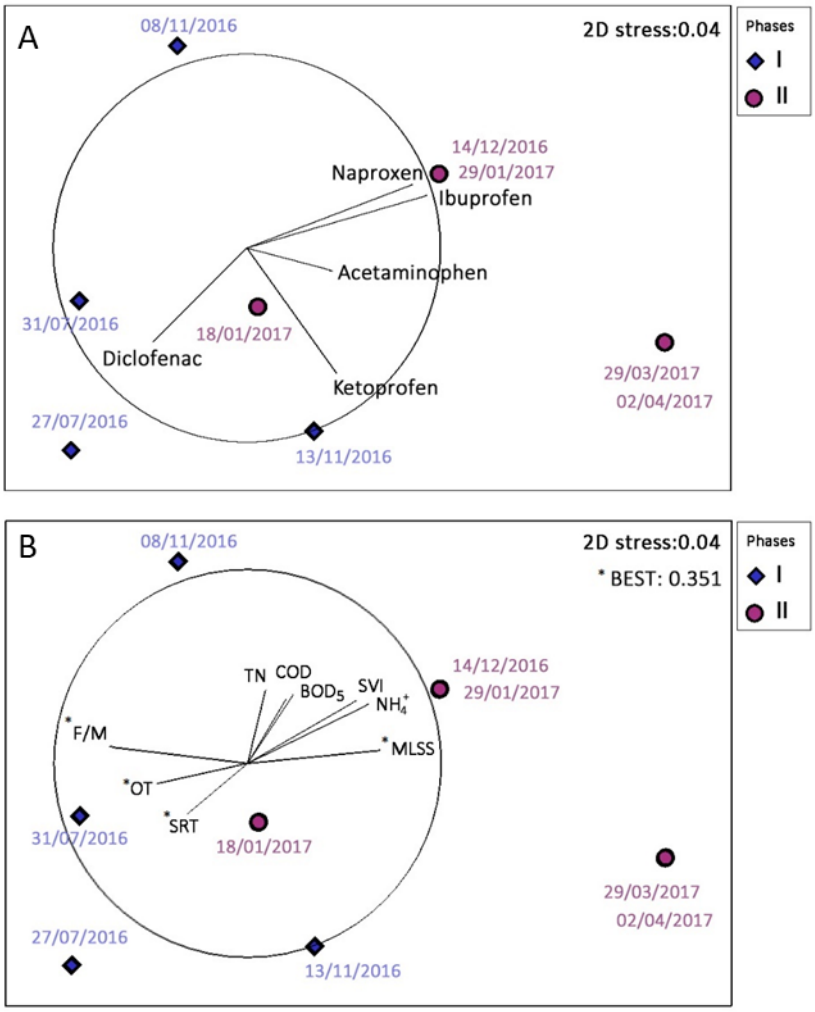
MDS multivariate analysis was used to infer the links among the REs of the 5 AIAPs measured at mean concentrations >1000 ngL^{-1} in the influent wastewater in both experimental phases (acetaminophen, ibuprofen, naproxen, ketoprofen, and diclofenac, Table 3) and the environmental/operational variables influencing the A^2O system (Figure 2). According to the results of BEST analysis, MLSS, F/M, SRT and OT contributed to explain 35% of the ordination of samples. The REs of naproxen, ibuprofen and acetaminophen were favored by the increased concentration of MLSS and the lower values of OT, SRT and F/M. RE of diclofenac displayed an opposite behavior, since the removal of this compound was higher in phase I compared to phase II. Finally, ketoprofen RE was mainly influenced by the shorter average F/M in phase II.

The A^2O system was operated using SRT longer than 10 days in both experimental phases (Table 1). According to the previous reports available, high RE values are often observed for ibuprofen and naproxen in WWTP designed for biological nutrient removal and operated with typical SRTs between 10 and 20 days (Jelic et al., 2011). When long SRTs are combined with high MLSS and low F/M, the increase of microbial biodiversity and the rates of organic compounds removal are favored, even at short HRTs (Verlicchi et al., 2012), in agreement with the observed improvement of REs of naproxen, ibuprofen and

Table 5. Removal efficiencies (%) of anti-inflammatory/analgesic pharmaceuticals (AIAPs) and relative standard compound in the experimental phases I (n=4) and II (n=5) in the A^2O system. The Kruskal Wallis test ($p<0.05$) was us of those AIAPs marked by an asterisk (*) was significantly different in the two phases.

	% REMOVAL EFFICIENCY					
AIAPs	Phase I					
	Range	Mean	Median	RSD (%)	n	Range
Acetaminophen	83 - 100	94	99	9	4	98 - 100
Ibuprofen *	17 - 60	39	40	60	4	43 - 100
Naproxen *	1 - 68	41	47	76	4	58 - 95
Ketoprofen	-51 - 40	0	5	-	4	7 - 82
Diclofenac	-21 - 44	9	6	325	4	-35 - 3
Codeine	-51 - 100	50	100	176	3	12 - 100
Indomethacin	0 - 100	60	70	76	4	-67 - 100
Propyphenazone	-100 - 100	33	100	1048	3	-15 - 100

Figure 2. A Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the activated sludge samples from the A^2O system in the experimental phases I and II, according to the removal efficiencies (RE) of acetaminophen, ibuprofen, ketoprofen, naproxen and diclofenac. Vectors on plot A. represent the direction throughout the ordination of each microbial group quantified in the samples, and vectors on plot B. represent the strength and directional influence throughout the ordination of the abiotic variables measured in the A^2O system. The variables which best explained the distributions of the RE of the target compound according to BIO-ENV analysis are marked with an asterisk (*).



BOD5: biological oxygen demand at 5 days; COD: chemical oxygen demand;
F/M: food-to-microorganisms ratio; MLSS: mixed liquor suspended solids;
OT: operating temperature; TN: Total N; SRT: sludge retention time;
SVI: sludge volumetric index.

acetaminophen in phase II of the present study.

High RE variability for ketoprofen has been reported in several studies, which postulate that its removal by biotransformation/biodegradation and sorption is highly influenced by the operational conditions and the type of technology of the WWTP plant (Verlicchi et al., 2012; Luo et al., 2014). In the present study, the increase of ketoprofen RE in phase II was found mostly correlated to the decrease of the F/M, as occurred for

ibuprofen and naproxen, (Figure 2). Park et al. (2017) obtained higher ketoprofen REs (87%) in a pilot-scale anoxic/aerobic MBR system, compared with a full-scale WWTP A^2O system (60%). Verlicchi et al. (2012) also reported higher ketoprofen RE in a MBR compared to CAS (70% vs. 56%, respectively). Due to the retention of biomass by the membranes, MBR systems can operate at higher MLVSS concentrations (5000–15,000 mg L^{-1}) and lower F/M, compared to CAS and A^2O systems. Overall, a system alternating different redox conditions (anaerobic, anoxic and aerobic), DO > 2 mgL^{-1}, MLSS > 5000 mg L^{-1} and F/M < 0.21 kg BOD_5 kg $MLVSS^{-1}$ d^{-1} are reported to achieve the highest (>80%) RE for ketoprofen, as well as for ibuprofen and naproxen (Jelic et al., 2011; Park et al., 2017).

3.4. Absolute and relative quantifications of total Bacteria, Mycolata, total Archaea and fungal populations

Since biodegradation/biotransformation processes are the main removal mechanisms for AIAPs in WWTPs, the population dynamics of the major microbial groups in activated sludge were monitored throughout both experimental phases, quantifying their absolute abundances by qPCR (Figure 3). The Kruskal-Wallis test revealed significant differences of the absolute abundance of marker genes of total Bacteria, Mycolata, total Archaea (number of copies of 16S rRNA genes) and Fungi (number of copies of 18S rRNA genes) among phases I and II (Table S5A).

The absolute abundance of total Bacteria per L activated sludge was in the 10^{10} range in phase I, while one order of magnitude higher was detected in phase II (10^{11}) (Table S5A). Moreover, total Bacteria abundance remained stable throughout each experimental phase. In contrast, more variations of the abundance of total Archaea were detected throughout the two phases studied (Figure 3A). The abundance of the archaeal 16S rRNA genes ranged between 10^7 and 10^9 copies L^{-1} activated sludge, and they were significantly more abundant in phase I than in phase II (ca. one order of magnitude higher), opposite to the results found for the bacterial marker genes.

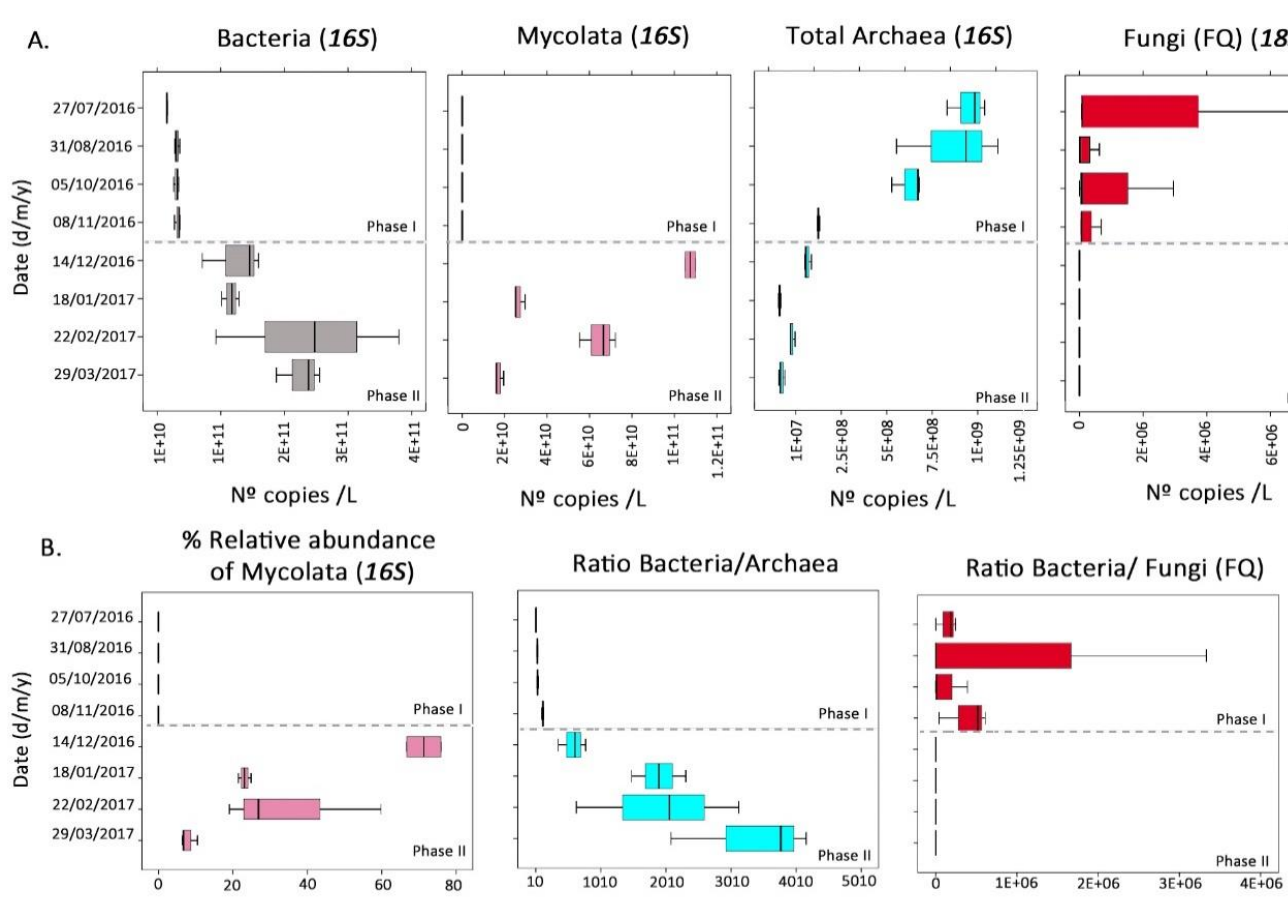
Mycolata were only detected during phase II and the numbers of their 16S rRNA copies per L activated sludge were kept in the 10^{10} order, while fungal populations were only detected in phase I. The abundance of fungal populations was evaluated in both experimental phases using two different primer sets (Table S3), due to the difficulties often associated to detect and quantify these eukaryotic microorganisms by molecular methods (Wurzbacher et al., 2014). Consistently, Fungi were detected only in phase I samples with

both sets of primers used, but the LR primer set provided quantifications nearly two orders of magnitude higher (ca.108 copies L^{-1}) than the FungiQuant primer set (ca.106 copies L^{-1}) (Table S5A) These differences could be due to PCR biases and differences in the coverage of the primer sets targeting different regions of the fungal 18S rRNA gene (Prévost-Bouré et al., 2011). Overall, the absolute abundances of total Bacteria, total Archaea, Mycolata and Fungi fell into the ranges described in previous works, performed in MBRs, moving-bed membrane bioreactors (MBMBR) or CAS treating urban wastewater (Gómez-Silván et al., 2014; Reboleiro-Rivas et al., 2015; Maza-Márquez et al., 2016; Niu et al., 2017).

Figure 3B and Table S5B display the relative abundances of Mycolata among the total Bacteria populations, as well as the total Bacteria/total Archaea and total Bacteria/Fungi ratios. Remarkably, Mycolata represented a high proportion of the total bacterial populations in activated sludge in phase II (average 31.02%). The highest relative abundance was detected on 14/12/2016, when the system operated with a SVI of 137 mgL^{-1} (Table 1). Several other authors previously stated that elevated SVI are strongly correlated with high abundances of Mycolata (Martins et al., 2004; Griffin and Wells, 2017).

Regarding the total Bacterial/total Archaea ratio, significant differences were detected between the experimental phases (10^1 versus 10^3, phases I and II, respectively). According to the literature, Bacteria are often more abundant than Archaea in CAS systems, and the Bacteria/Archaea ratios range from 1 to 10^5 (González-Martínez et al., 2017; Castellano-Hinojosa et al., 2018). The results of the present study suggest that outcompeting of Archaea by Bacteria was particularly favored under the experimental conditions of phase II. Negative interactions between Bacteria and Archaea in complex ecosystems have been previously reported (Steele et al., 2011). The average abundance of copies of total Bacteria 16S rRNA genes was also several orders of magnitude higher than the numbers of fungal 18S rRNA copies in phase I, even though the Bacteria/Fungi marker gene ratios varied depending on the fungal primer set chosen (10^5 for the FungiQuant primer set, and 10^3 for the LR primer set, Table S5B).

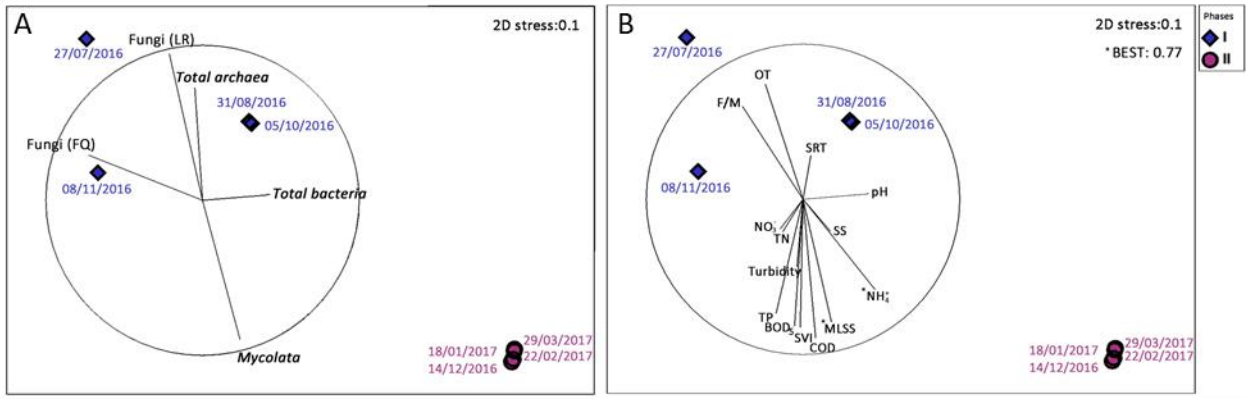
Figure 3. A. Box and whisker plots of the number of copies per L activated sludge of marker genes of total Bacter
quantified by qPCR throughout the experimental phases I and II in the A^2O system. Fungi were quantified using two di
and LR). In plots, upper and lower bounds of the box denote the 75^{th} and 25^{th} percentiles, and upper and lower bounds
B. Relative abundance of Mycolata, expressed as percentage of total Bacteria, and ratios of Bacteria/Archaea and
abundances and ratios are based on the qPCR quantifications in samples of the A^2O retrieved throughout two experime



3.5. Linking the abundance of total Bacteria, Mycolata, total Archaea and Fungi to the environmental/operational variables of the A^{2}Osystem

Activated sludge samples retrieved from the A^{2}O process were ordinated by MDS, on the basis of the qPCR quantifications of the absolute abundance of copies of marker genes of total Bacteria, Mycolata, total Archaea and Fungi (Figure 4). Divergent behavior trends were detected in the ordination for the abundances of the different targeted microbial groups. The absolute abundances of total Archaea and Fungi correlated negatively to the abundances of Mycolata ($r < -0.75$), while total Bacteria correlated negatively with Fungi ($r = -0.90$), but only when the later were quantified using the FungiQuant primer set (Figure 4, Table S6).

Figure 4. Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the activated sludge samples from the A^{2}O system in the experimental phases I and II, according to the abundance of copies of marker genes of total Bacteria, Mycolata, total Archaea and Fungi. Fungi were quantified using two different set of primers (FungiQuant (FQ) and LR). Vectors on plot **A.** represent the direction throughout the ordination of each microbial group quantified in the samples, and vectors on plot **B.** represent the strength and directional influence throughout the ordination of the abiotic variables measured in the A^{2}O system. The variables which best explained the distributions of the biological data according to BIO-ENV analysis are marked with an asterisk (*)



BOD_5: biological oxygen demand at 5 days; COD: chemical oxygen demand; F/M: food-to-microorganisms ratio; MLSS: mixed liquor suspended solids; OT: operating temperature; TN: total N; SRT: sludge retention time; SVI: sludge volumetric index

The vectors representing the environmental/operational variables influencing the A^{2}O system displayed two clear trends throughout the MDS ordination, according to the differences among the two experimental phases. OT and F/M values increased in the direction of phase I samples, while the rest of physicochemical variables pointed in the opposite direction to phase II samples. According to BIO-ENV analysis, concentrations of MLSS in the sludge and N-NH_4^+ in the influent wastewater were the parameters

contributing the best explanation (77%) to the overall ordination of samples (Figure 4). Besides, strong directional influences of OT, F/M, and organic load of the influent water (COD, BOD_5) were observed on the ordination (length of vectors > 0.8, Figure 4). Positive correlations were found between F/M, OT and the abundance of Fungi and Archaea ($r > 0.60$), while Mycolata abundance was positively correlated with MLSS ($r = 0.99$), COD, and N-NH_4^+ concentration ($r > 0.80$) (Figure 4, Table S6). The results described here indicate that physicochemical conditions and operational parameters exerted significant influences on the abundance of the different major microbial groups in the A^2O system, in agreement with previous works in WWTPs (Reboleiro-Rivas et al., 2015; Maza-Márquez et al., 2016; Weithmann et al., 2016; Griffin and Wells, 2017).

Phase II was operated with the lowest average values of F/M and OT, while the organic load was 35% higher and the concentration of MLSS nearly twofold than in phase I (Table 1). Higher C availability favors fast-growing heterotrophic bacteria, leading to increased MLSS, and accordingly, the number of total Bacteria was one order of magnitude higher in phase II. Previous researchers have also described that an increase of MLSS concentration correlates with higher numbers of heterotrophic bacteria in WWTPs (Molina-Muñoz et al., 2010; Kimet al., 2011; Reboleiro-Rivas et al., 2015).

Mycolata are Gram-positive microorganisms frequently found in activated sludge in WWTPs (Guo and Zhang, 2012; Fang et al., 2013; Maza-Márquez et al., 2015). Although this group comprises both fast and slow-growing bacteria and are known to proliferate under a wide range of operational conditions in WWTPs (de los Reyes, 2010), they were detected by qPCR only during phase II of the present study. According to BIO-ENV analysis, OT, F/M, organic load and MLSS were variables with strong influence on the abundance of Mycolata in the A^2O system ($r > \pm 0.85$, Table S6). These factors are also well documented to significantly affect Mycolata occurrence and population dynamics in WWTPs in the earlier literature (de los Reyes, 2010; Asvapathanagul et al., 2012; Parada-Albarracín et al., 2012; Maza-Márquez et al., 2015). The proliferation of Mycolata was favored by the combination of environmental/operational variables during phase II (higher concentrations of substrates in the influent water and MLSS, lower OT and lower F/M, Table 1), and also correlated positively ($r = 0.98$) with SVI (Figure, Table S6); however, no bulking/foaming episodes took place after the transition period among phases I and II. Other authors have observed that high densities of Mycolata can occur in the absence of bulking/ foaming in MBRs (Parada-Albarracín et al., 2012). Since Mycolata are a

heterogeneous group displaying wide phylogenetic and physiological diversity, strains highly vary in their ability to exhibit foaming capacity (Petrovski et al., 2011).

The removal of AIAPs in the A^2O system was improved in experimental phase II for 4 of the 5 most abundant compounds measured in the influent wastewater, with statistically significant higher REs for ibuprofen and naproxen (Table 3 and 5), indicating that the succession of microbial communities in the reactor due to the changes of the operational and physicochemical conditions positively influenced their removal performance. Fungi were not detected during phase II, while total Bacteria increased one order of magnitude and Mycolata were detected in high relative abundances among them (Figure 3). Under such conditions, we hypothesize that Mycolata were likely involved in the improved removal of the analyzed AIAPs in phase II. Actinobacteria are nutritionally flexible microorganisms, known to play important roles in the degradation of organic pollutants in WWTPs (Nielsen et al., 2009; de los Reyes, 2010; Maza-Márquez et al., 2015), including PhACs (Pala-Ozkok et al., 2014; Zhu et al., 2015; Zhang et al., 2016; Jiang et al., 2017). Among Actinobacteria, Mycolata display versatile metabolic capabilities and assimilate both hydrophilic and hydrophobic substrates (Nielsen et al., 2009). Microbial transformation of ibuprofen has been characterized in *Nocardia iowensis* strain NRRL 5646, isolated from soil (Chen and Rosazza, 1994). In a more recent study, Almeida et al. (2013) screened bacterial isolates from activated sludge samples in search of strains able to biodegrade ibuprofen or ketoprofen in pure culture, reporting that 50% of the selected strains were phylogenetically related to Mycolata (*Gordonia* spp., *Tsukamurella* and *Rhodococcus*). Three isolates identified as *Gordonia amicalis*, *G. hydrophobica* and *Tsukamurella pumalis* displayed REs of 26% for ibuprofen, 22% and 21% for ketoprofen, respectively. Remarkably, the average RE of ketoprofen was higher on phase II in the A^2O system analyzed in the present study, while the REs of this compound were often negative during phase I, in absence of detectable numbers of Mycolata.

The correlations among the RE of AIAPs, the environmental/operating variables influencing WWTPs and the abundances of the major microbial groups in activated sludge have been seldom explored to date. In this respect, future work aiming to monitor community structure and diversity changes in response to abiotic variables favoring AIAPs removal is planned, in order to elucidate the role of the complex microbial community of activated sludge in the removal of these PhACs in WWTPs.

4. Conclusion

The results of the present study show that environmental/operating variables influencing the A^2O system significantly influenced both the RE of AIAPs and the occurrence and abundance of total Bacteria, Mycolata, total Archaea and Fungi; however, in spite of microbial abundance variability, dramatic changes of the stability and resilience of the A^2O system were not observed. Improved REs of several of the AIAPs analyzed (acetaminophen, ibuprofen, naproxen, ketoprofen) were correlated to higher organic load in the influent water (COD, BOD_5), higher concentration of MLSS, lower OT and lower F/M. These factors also favored the occurrence and abundance of Mycolata in activated sludge, pointing at this group of bacteria as candidate key players for AIAPs removal in activated sludge.

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Supplementary material for Chapter 1:

"Removal of anti-inflammatory/analgesic drugs from urban wastewater in a pilot-scale A^2O system: linking performance and microbial population dynamics to operating variables"

1. List of chemicals used (pharmaceutical standards and surrogates) and brands

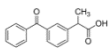
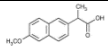
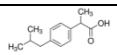
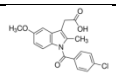
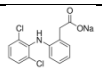
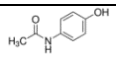
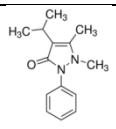
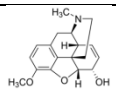
All pharmaceutical standards were of high purity grade (>90%). Ketoprofen was supplied by Jescuder (Rubí, Spain). Naproxen, Indomethacin, Diclofenac (as sodium salt) and Propyphenazone were purchased from Sigma-Aldrich (Steinheim, Germany). Ibuprofen, Acetaminophen, and Codein were from Cerilliant (Sigma Aldrich, Texas, USA).

Isotopically labelled compounds, used as surrogates, were ketoprofen-$^{13}CD_3$ and acetaminophen d_4, purchased from Sigma Aldrich (Steinheim, Germany). Codeine-d_3 was purchased from ISOTEC (Sigma Aldrich, Steinheim, Germany). Ibuprofen-d_3 was supplied by CDN Isotopes (Quebec, Canada).Naproxen-d_3, indomethacin-d_4, diclofenac-d_4, and carbamazepine-d_{10}, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA)

Table S1. Compounds and their optimized QqLIT-MS/MS parameters by SRM negative and positive ionization mode a

Compounds	Precursor ion (m/z)	SRM 1	DP-CE-CXP	SRM 2	DP-CE-CXP	Rt (min)	SRM /SRM
Ketoprofen	253.0 $[M-H]^-$	209	40-12-11	197	40-6-9	8.1	38.
Ketoprofen-13C-d3 (S)	257.0 $[M-H]^-$	213	40-12-11	-	-	8.1	-
Naproxen	229.0 $[M-H]^-$	169	35-38-9	185	35-10-13	8.6	1
Naproxen-d3 (S)	232.0 $[M-H]^-$	188	35-10-13	-	-	8.5	-
Diclofenac	294.0 $[M-H]^-$	250	40-16-1	214	40-30-15	10.5	12.
Diclofenac-d4 (S)	297.6 $[M-H]^-$	254.4	35-16-13	-	-	10.4	-
Indomethacin	356.0 $[M-H]^-$	312	50-12-3	297	50-24-17	11.1	3.3
Indomethacin-d4 (S)	360.0 $[M-H]^-$	316	50-12-3	-	-	11.1	-
Ibuprofen	205.0 $[M-H]^-$	161	45-10-7	-	-	11.3	-
Ibuprofen-d3 (S)	208.0 $[M-H]^-$	164.2	25/10/2005	-	-	11.3	-
Acetaminophen	150.0 $[M-H]^-$	110	76-23-0	93	61-21-6	4.3	6.3
Acetaminophen-d4 (S)	154.3 $[M+H]^-$	113.9	46-23-6	-	-	3.5	-
Codeine	300.0 $[M+H]^+$	152	106-85-12	115	106-105-6	4.6	1.2
Codeine-d3 (S)	303.2 $[M+H]^+$	115	46.105-8	-	-	4.6	-
Propyphenazone	231.0 $[M+H]^+$	56	36-57-4	189	36-35-16	11.2	1.3
Carbamazepine-d10 (S)	247.0 $[M+H]^+$	204	71-31-16	-	-	10.3	10.

Table S2. Target compounds by therapeutic groups with surrogates.

Compound	Structure	CAS Number	Molecular formula	Molecular weight	Precursor ion (*m/z*)	MRM (m/z) Quantification
Ketoprofen		22071-15-4	$C_{16}H_{14}O_3$	254.281	253.0 $[M-H]^-$	209
Naproxen		22204-53-1	$C_{14}H_{14}O_3$	230.263	229.0 $[M-H]^-$	169
Ibuprofen		15687-27-1	$C_{13}H_{18}O_2$	206.285	205.0 $[M-H]^-$	161
Indomethacin		53-86-1	$C_{19}H_{16}ClNO_4$	357.788	356.0 $[M-H]^-$	312
Diclofenac		15307-86-5	$C_{14}H_{11}C_{l2}NO_2$	296.148	294.0 $[M-H]^-$	250
Acetaminophen		103-90-2	$C_8H_9NO_2$	151.163	150.0 $[M-H]^-$	110
Propyphenazone		479-92-5	$C_{14}H_{18}N_2O$	230.305	231.0 $[M+H]^+$	56
Codeine		76-57-3	$C_{18}H_{21}NO_3$	299.364	300.0 $[M+H]^+$	152

[a]Internal Standard differs from original method.

Table S3. A. Primer pairs used for the quantification of total Bacteria, total Archaea, filamentous bacteria (Mycolata) a
for the quantification of the target marker genes of each microbial group.

A.

Group	Primer sequence (5´-3´)	Gene marker	Amplicon size (base pairs, bp)
Total Bacteria			
341F	CCTACGGGAGGCAGCAG	16S rRNA	194
534R	ATTACCGCGGCTGCTGG		
Total Archaea			
UNI-b-rev (R)	GACGGGCGGTGTGTRCAA	16S rRNA	456
ARCH915 (F)	AGGAATTGGCGGGGGAGCAC		
Mycolata			
MycolataF249	GATGGGCCCGCGGCCTATCA	16S rRNA	442
MycolataR689	TACACCAGGAATTCCAGTCT		
Fungi			
FungiQuantF	GGRAAACTCACCAGGTCCAG	18S rRNA	351
FungiQuantR	GSWCTATCCCCAKCACGA		
LR3 (forward)	CCGTGTTTCAAGACGGG	18S rRNA	625
LROR (reverse)	ACCCGCTGAACTTAAGC		

B.

		Total Bacteria	**Total Archaea**	**Mycolata**	**Fungi** **Fungi**
	Initial denaturalization	95°C, 7 minutes	95°C, 7 minutes	94°C, 7 minutes	95°C,
Amplification (×40 cycles)	Denaturalization	95°C, 30 seconds	95°C, 30 seconds	94°C, 60 seconds	94°C,
	Primers annealing	60°C, 40 seconds	65°C, 30 seconds	58°C, 60 seconds	62°C,
	Elongation	72°C, 30 seconds	72°C, 30 seconds	72°C, 30 seconds	72°C,
	Melting curve	60°C-95°C + 2°C/minute. Fluorescence measured			
	Hold	25°C			

Table S4. Anti-inflammatory/analgesic drugs (AIAPs) consumed in Spain in year 2016, in terms of **DHD** (Daily doses defined per 1000 inhabitant per day), **DDD** (Daily Doses Defined, mg active compound/d). Excretion factor **(E)**, and the predicted environmental concentration in the influent (**PEC,** ng/L) are included too.

AIAPs	**DDD (mg d^{-1})**	**DHD (DDD 1000 inh^{-1} d^{-1})**	**E**	**PEC influent (ng L^{-1})**
Acetaminophen	3000	15.00	0.8[a]	159840
Ibuprofen	1200	13.67	0.15[b]	10925
Naproxen	1200	9.23	0.05[a]	2458
Ketoprofen	150	3.02	0.8[b]	1609
Diclofenac	100	3.25	0.39[c]	563
Codeine	100	0.51	n.a.	226*
Indomethacin	100	0.30	n.a.	133*
Propyphenazone	3000	0.01	n.a.	133*

[a]Verlicchi et al. (2012).
[b]Jelic et al. (2015).
[c]Al Aukidy et al. (2012).
n.a. data not available.
* Value calculated with 100 % excretion factor.

Table S5. A. Mean ± standard deviations of the numbers of copies per L activated sludge of total Bacteria, tot
populations, quantified by qPCR in the A^2O system. Fungi were quantified using two different set of primers
differences between phases I and II were detected for total Bacteria and Archaea abundances, according to th
Relative abundances (average ± standard deviations), expressed as ratios to total Bacteria, total Archaea, Myc
system. Fungi were quantified using two different set of primers (FungiQuant and LR). Significant difference
detected for Archaea relative abundances, according to the Kruskal-Wallis test ($p<0.05$). BLD: below the lim

A.

	Total Bacteria	**Total Archaea**	**Mycolata**	**Fungi (FungiQuan**
Phase I	$2.74 \times 10^{10} \pm 7.94 \times 10^{9}$	$7.92 \times 10^{8} \pm 3.72 \times 10^{8}$	BLD	$1.43 \times 10^{6} \pm 1.12 \times$
Phase II	$1.77 \times 10^{11} \pm 6.55 \times 10^{10}$	$1.19 \times 10^{8} \pm 6.98 \times 10^{7}$	$5.31 \times 10^{10} \pm 3.94 \times 10^{10}$	BLD

B.

	Mycolata	**Total Archaea**	**Fungi (FungiQuant)**
Phase I	BLD	51.1 ± 46.2	$2.44 \times 10^{5} \pm 1.29 \times 10^{5}$
Phase II	39.4 ± 36.5	$1.94 \times 10^{3} \pm 1.12 \times 10^{3}$	BLD

Table S6. Pearson's product-moment correlation coefficients between the absolute abundances of total Bacteria, Mycolata, total Archaea and Fungiper L activated sludge samples (quantified by qPCR) and the environmental variables and operational parameters influencing the A^2O system. Fungi were quantified using two different set of primers (FungiQuant (FQ) and LR). Strong correlations ($r \geq 0.50$) are boldfaced. (*).BOD_5: biological oxygen demand at 5 days; COD: chemical oxygen demand; DO: dissolved oxygen; F/M: food-to-microorganisms ratio; MLSS: mixed liquor suspended solids; OT: operating temperature; TN: Total N; TP: total P; TSS: total suspended solids in influent water; SRT: sludge retention time; SVI: sludge volumetric index.

	Total Bacteria	Mycolata	Total Archaea	Fungi (FQ)	Fungi (LR)
MLSS	0.14	**0.99**	**-0.99**	**-0.57**	**-1.00**
SVI	-0.10	**0.98**	**-0.99**	-0.35	**-0.97**
OT	-0.23	**-1.00**	**0.97**	**0.64**	**1.00**
pH	**1.00**	0.31	0.01	**-0.90**	-0.14
F/M	-0.47	**-0.97**	**0.88**	**0.82**	**0.94**
SRT	0.25	**-0.71**	**0.97**	0.20	**0.92**
Turbidity	-0.17	**0.87**	**-0.99**	-0.29	-0.95
TSS	**0.58**	**0.91**	**-0.81**	**-0.88**	**-0.89**
BOD_5	-0.15	**0.87**	**-0.99**	-0.31	**-0.96**
COD	0.01	**0.98**	**-1.00**	-0.45	**-0.99**
Total N	**-0.60**	**0.56**	**-0.81**	0.17	**-0.72**
$N\text{-}NO_3^-$	**-0.67**	**0.51**	**-0.75**	0.27	**-0.64**
$N\text{-}NH_4^+$	**0.55**	**0.85**	-0.05	0.27	**-0.64**
Total P	-0.31	**0.83**	**-0.96**	-0.15	**-0.90**
Fungi (LR)	-0.14	**-0.97**	**0.99**	**0.57**	-
Fungi (FQ)	**-0.90**	**-0.76**	0.43	-	-
Total Archaea	0.01	**-0.92**	-	-	-
Mycolata	0.31	-	-	-	-

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❖ CHAPTER 2

Linking microbial diversity and population dynamics to the removal efficiency of pharmaceutically active compounds (PhACs) in an anaerobic/anoxic/aerobic (A^2O) system

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Abstract

The removal efficiencies (REs) of nineteen pharmaceutically active compounds (PhACs) (six antibiotics-clarithromycin, ofloxacin, sulfadiazine, sulfamethazine, sulfamethoxazole and trimethoprim -, four β-blockers -atenolol, metoprolol, propranolol and sotalol- two antihypertensives/diuretics -furosemide and hydrochlorothiazide-, three lipid regulators -bezafibrate, fenofibrate and gemfibrozil-, and four psychiatric medications -carbamazepine, diazepam, lorazepam and paroxetine) were ascertained in a pilot-scale anaerobic/anoxic/aerobic (A^2O) system treating urban wastewater, long term operated during two experimental phases using different sets of environmental conditions and operating parameters. Illumina MiSeq sequencing was used to investigate the structure, diversity and population dynamics of bacteria, archaea and fungi communities in the activated sludge. The results showed that mixed liquor suspended solids (MLSS) and food-to-microorganisms ratio (F/M) were operational parameters significantly influencing the REs of five of the analyzed PhACs in the A^2O system. Biota-environment (BIO-ENV) analysis revealed strong correlations between population shifts of the activated sludge community and the REs of PhACs of the different pharmaceutical families. Increased REs of clarithromycin, furosemide, bezafibrate and gemfibrozil were concomitant to higher relative abundances of bacterial phylotypes classified within the *Rhodobacteraceae* and *Sphingomonadaceae* (*Alphaproteobacteria*), while those of *Betaproteobacteria*,

Chloroflexi and *Methanomethylovorans* (*Euryarchaea*) correlated positively with the REs of up to seven PhACs belonging to different therapeutic groups.

1. Introduction

The occurrence and fate of contaminants of emerging concern in aquatic environments and their potential ecotoxicological effects are current issues which have gained relevance in the last decade. Among such contaminants, pharmaceutically active compounds (PhACs) bring forward a special interest among the population (Al Aukidy et al., 2012; Verlicchi et al., 2012). PhACs of different therapeutic classes such as antibiotics, anti-inflammatory and/or analgesic substances, β-blockers, diuretics, lipid regulators or psychiatric medications, occur in raw wastewaters at varying concentrations ranging from ng L^{-1} to μg L^{-1}, depending on the prescription habits and usage patterns of each particular country (Kasprzyk-Hordern et al., 2009; Petrie et al., 2015). Among these, anti-inflammatory and/or analgesic pharmaceuticals (AIAPs) such as acetaminophen, ibuprofen or naproxen and the lipid regulators bezafibrate, fenofibrate and gemfibrozil are most commonly detected and at the highest concentrations, reaching values up to several hundred μg L^{-1} (Miège et al., 2009; Tran et al., 2018). Global consumption of PhACs and consequently their incidence in wastewaters are predicted to increase rather than decay in the near future, due to the lengthening of life expectancy of the population (Van Der Aa et al., 2011).

Biodegradation and sorption onto solids are assumed to be the primary mechanisms for the removal of the majority of PhACs in wastewater treatment plants (WWTPs), but the removal rates may considerably differ for each particular compound depending on its chemical characteristics and are also influenced by the operating conditions of the treatment systems (Pin Gao et al., 2012; Jelić et al., 2011; Petrovic et al., 2009; Radjenović et al., 2009). In most cases, the biodegradation of these substances is determined by the presence of primary carbon or nitrogen substrates, as well as the existence of microorganisms implicated in cometabolic degradation (Tran et al., 2013). Nevertheless, WWTPs have been shown unable to completely remove most of the PhACs present in the influent wastewaters and consequently many of these substances are detected in the treated effluents and stabilized sewage sludge. For example, β-blockers such as atenolol, metoprolol, propranolol, the antiepileptic drug carbamazepine, the AIAP diclofenac, and antibiotics belonging to several chemical classes such as fluoroquinolones, macrolides or sulfonamides, are detected both in influents and effluents of WWTPs worldwide (Gurke et al., 2015; Kasprzyk-Hordern et al., 2008, 2009; Tran et al., 2018; Verlicchi et al., 2012).

In addition, the microbial community of activated sludge comprises a high density and diversity of bacterial populations subjected to organic pollutants stresses, making WWTPs optimal scenarios for the spreading of antibiotic resistances and posing important risks for human health due to the transfer of the antibiotic resistance genes (ARGs) from environmental reservoirs to human pathogenic bacterial strains (Hultman et al., 2018). Last, but not least, the presence of antibiotics in wastewaters may affect the diversity and functionality of the complex microbial communities in charge of the biological wastewater treatment, consequently hampering their performance (Deng et al., 2012; Rodriguez-Sanchez et al., 2017).

The concentration of PhACs in treated water often exceeds *the predicted no effect concentrations* (PNECs) for aquatic and soil organisms, so the development of more efficient wastewater treatment configurations with the aim to reduce their concentrations in the treated effluents is a requisite to prevent the dissemination of these substances to water and soil bodies (Kümmerer, 2009). In this sense, several biological treatment technologies (as alternative to the conventional activated sludge system, CAS) have been evaluated with regard to their efficiency for PhACs removal: membrane bioreactors (MBR), fixed bed bioreactors (FBR), moving bed biofilm reactors (MBBR), aerobic or anaerobic granular sludge (AGS, AnGS), and anaerobic/anoxic/aerobic (A^2O) systems (Lv et al., 2017; Park et al., 2017). However, few studies have attempted to relate the removal efficiencies (REs) of PhACs to the shifts in the structure and diversity of the microbial communities of activated sludge, which in turn are linked to changes of the operational parameters and other uncontrolled variables influencing the WWTPs under real operation conditions.

In this study, the removal efficiencies of nineteen PhACs (six antibiotics-clarithromycin, ofloxacin, sulfadiazine, sulfamethazine, sulfamethoxazole and trimethoprim-, four β-blockers -atenolol, metoprolol, propranolol and sotalol- two antihypertensives/diuretics -furosemide and hydrochlorothiazide-, three lipid regulators -bezafibrate, fenofibrate and gemfibrozil-, and four psychiatric medications -carbamazepine, diazepam, lorazepam and paroxetine) were evaluated in a pilot-scale A^2O system. The A^2O technology was selected for this study, since this advanced wastewater treatment process is based on a single sludge configuration using a combination of anaerobic, anoxic and aerobic bioreactors in series, which provides better removal efficiency of PhACs compared to CAS (Grandclément et al., 2017; Verlicchi et al., 2012). The A^2O was long-term operated during two experimental phases, using different operation parameters and under different environmental conditions. The Illumina MiSeq sequencing

platform was used to investigate the structure, diversity and population dynamics of bacteria, archaea and fungi, in order to find significant links among the observed variability of the relative abundances of microbial populations, the changes of the environmental/operational variables, and the REs of the targeted PhACs, with the aid of multivariate statistical analyses.

2. Materials and methods

2.1. Description of the wastewater treatment pilot-scale plant and operational conditions

The pilot-scale A^2O plant (Figure 1 of Chapter 1) was located at the facilities of the WWTP *Murcia Este* (EMUASA, Murcia, Spain) and its characteristics have been previously described in full detail (Gallardo-Altamirano et al., 2018). In brief, the working period covered from May 20th 2016 to March 31th 2017, divided into two differentiated experimental phases: phase I (July 27th - November 13th 2016) and phase II (December 14th 2016 - March 31th 2017), which lasted 104 and 105 days, respectively, separated by a transition phase needed for the system to reach stable operation under the parameters chosen for phase II. Average values ± standard deviations for operational parameters of the A^2O system and physical-chemical parameters measured in the influent water samples during phases I and II are summarized in Table S1.

2.2. Wastewater sampling collection for the analysis of physicochemical parameters and PhACs

The A^2O system was sampled at points 1, 2 and 3, as shown in Figure 1 of Chapter 1. Daily-composite samples were retrieved three times a week. Chemical oxygen demand (COD), total nitrogen (TN), $N\text{-}NO_3^-$, $N\text{-}NH_4^+$ and total phosphorous (TP) were measured with the aid of Merck Spectroquant® kits (Darmstadt, Germany), while biological oxygen demand (BOD_5), total suspended solids in the influent water (TSS), Mixed liquor suspended solids (MLSS) and volatile suspended solids (MLVSS) were measured according to standard methods (Baird and Bridgewater, 2017).

For the analysis of PhACs, eighteen influent and effluent wastewater 24-h composite samples (eight samples in phase I and ten samples in phase II) were collected from sampling points 1 and 3 of the pilot scale plant (Figure 1 of Chapter 1), using 500-mL amber PET bottles as described previously (Gallardo-Altamirano et al., 2018). Samples

were retrieved both midweek and at the end of the weekend and were frozen at -20 °C until their analysis. The effluent samples were collected according to the constant HRT (12 h).

2.3. Analytical methods for pharmaceutically active compounds

2.3.1. Chemicals and sample treatment

Analytical reference standards (mostly 90% purity, Table S2A) and isotopically labelled compounds for internal standard calibration (Table S2B) were purchased from Sigma-Aldrich (St Louis, MO, USA), Cerilliant (Round Rock, TX, USA), CDN Isotopes (Quebec, Canada), LGC Promochem (London, UK), and Santa Cruz Biotechnology (Dallas, TX, USA). Individual stock solutions of the standards were prepared in MeOH at a concentration of 100 µg mL^{-1}. For calibration and spiking purposes, working solutions (10 µg mL^{-1} in MeOH) including all tested compounds or labelled compounds were freshly prepared every three months. All solutions were stored in the dark at -20°C. HPLC-grade solvents (MeOH, ACN, and water) were purchased from Merck (Darmstadt, Germany).

2.3.2. On-line extraction and LC-MS/MS analysis

Pharmaceutical compounds were extracted from wastewater samples using methods previously validated and reported elsewhere (Gros et al., 2009; López-Serna et al., 2010). Briefly, 2.5 mL of sample were pre-concentrated onto a previously conditioned HySphere Resin GP 10 online-SPE cartridge (Spark Holland, Emmen, The Netherlands) and analysed using an automated on-line SPE sample processor Symbiosis™ Pico (Spark Holland) connected in series with a 4000 QTRAP hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer (Sciex, Redwood City, CA, U.S.). An exhaustive extraction procedure was detailed in Gallardo-Altamirano et al. (2018).

The retained analytes were eluted from the cartridge with the LC mobile phase (linear organic gradient of acetonitrile in water with formic acid for positive ionization and ACN:MeOH in water for negative ionization) and transferred to the LC column (Purospher STAR RP-18 endcapped column (125 x 2 mm i.d., 5 µm particle size, Merck, Darmstadt, Germany). The 4000 QTRAP hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer, equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, California, USA), was controlled by the Analyst 1.4.2 Software (Applied Biosystems-Sciex, Foster City, California, USA).

Ionization of the analytes was done using a Turbo Ion Spray source operated in both positive and negative electrospray ionization mode. The negative ionization mode allowed

detection of four pharmaceuticals (bezafibrate, furosemide, gemfibrozil and hydrochlorothiazide) including isotopically-labeled compounds. In positive ionization mode, fifteen compounds (atenolol, carbamazepine, clarithromycin, diazepam, fenofibrate, lorazepam, metoprolol, ofloxacin, paroxetine, propranolol, sotalol, sulfamethoxazole, and trimethoprim) were analyzed including isotopically-labeled compounds. For quantitative analysis, the ESI-MS/MS methods were performed, as described by Gros et al. (2009). MS acquisition was performed in the selective reaction monitoring (SRM) mode, by acquiring two SRM transitions per each target compound and only one for the corresponding surrogate compound. Target compounds and surrogates are listed in Table S3. Selected SRM transitions for each analyte and isotopically-labelled compound and the optimal instrumental conditions set for their analysis are summarized in Table S4**.**

2.4. DNA extraction, Illumina Miseq sequencing and data processing

Genomic DNA from 4-ml activated sludge samples was extracted as previously described (Gallardo-Altamirano et al., 2018). The genomic DNA pools were sequenced at the facilities of RTL Genomics (Lubbock, Texas, USA, http://www.researchandtesting.com), using an Illumina MiSeq platform (Illumina, Hayward, CA, USA). The primer pairs 28F-519R (5'-GAGTTTGATCNTGGCTCAG-3' and 5'-GTNTTACNGCGGCKGCTG-3') (Fan et al., 2012), 517F/909R (5'-GCYTAAAGSRNCCGTAGC-3' and 5'-CCCCGYCAATTCMTTTRAGT-3) (Maspolim et al., 2015b) and FungiQuantF/FungiQuantR (5-GGRAAACTCACCAGGTCCAG-3' and 5'-GSWCTATCCCCAKCACGA-3') (Liu et al., 2012) were chosen for the partial amplification of the 16S rRNA gene of bacteria and archaea and the18S rRNA gene of fungi, respectively.

Raw sequencing data were processed using the QIIME software, v. 1.9.1, following the pipeline protocol previously described by Caporaso et al. (2012). The sequences underwent a quality filtering as previously described by Bang-Andreasen et al. (2017), which removed low-quality reads (Q score ≤ 25), reads with homopolymers longer than 6 nt, ambiguous nucleotides (n ≥ 6), or consecutive low-quality base calls (n ≥ 5). Chimeric sequences were removed with USEARCH (Edgar, 2010). Finally, single sequence reads occurring in only one sample were removed of the PHY_table_BIOM for further analyses. Taxonomic *close-reference* assignments based on 97% sequence identity were done with QIIME v. 1.9.1 (Caporaso et al., 2010) using the Greengenes database (version 13_08) for Bacteria and Archaea sequences, while the 18S rRNA eukaryotic SILVA database (version 111) was used for Fungi (Yarza et al., 2017). The descriptive alpha-diversity indices Chao-

1, Shannon-Wiener *H'*, and Simpson were calculated using QIIME. Good's coverage indices were also calculated as described previously (Good, 1953). Sample-size-based and coverage-based rarefaction and extrapolation (R/E) sampling curves with 95% confidence intervals were calculated and plotted using the 'iNEXT' free online tool (Chao et al., 2016; Hsieh et al., 2016), in order to make fair comparisons among samples of unequal size. Heatmaps displaying the relative abundances of bacteria, archaea and fungi in each sample were generated using the vegan package in R v.3.2.0 (http://www.r-project.org). The SIMPROF and SIMPER commands of the Primer software (PRIMER-E v. 6.1.18, Plymouth, UK) were used to construct an UPGMA dendrogram with 95% confidence intervals and to estimate the contribution of individual PHYs to the (dis)similarity between groups of samples, respectively, following the methods already described by Maza-Márquez et al. (2016).

2.5. Statistical analyses

IBM SPSS Statistics v. 19 (SPSS Inc., IBM, USA) was used to perform the statistical analyses on the different data sets. The Kruskal-Wallis non-parametric test was selected to find significant differences among the different groups of samples, using a 95% significance level ($p < 0.05$). When required, Spearman's rank correlation coefficients (ρ) among the different variables were calculated.

All the multivariate statistical analyses were performed using the Primer software (PRIMER-E v. 6.1.18, Plymouth, UK). Non-metric multidimensional scaling (MDS) was used for the ordination of activated sludge samples based on the biotic data sets (relative abundances of PHYs of the targeted groups, derived from the Illumina-sequencing platform). An analysis of similarity (ANOSIM) was also conducted. The ordination of samples was linked to the trends followed by the abiotic variables (REs of the targeted PhACs and environmental/operational variables influencing the A^2O system) by means of BIO-ENV (biota-environment) analysis. Full details are described elsewhere (Maza-Márquez et al., 2016b).

3. Results and discussion

3.1. Occurrence of PhACs in influent and effluent wastewater samples.

Table 1 and 2 display the frequency of detection and the concentrations (range, mean, median and relative standard deviation, RSD) of the 19 targeted PhACs in the influent wastewater samples and the effluents of the A^2O system during the experimental phases I

and II. As shown in Table 1, 13 compounds had ≥ 75% frequency of detection in the influent wastewater in at least one of the experimental phases, of which clarithromycin, ofloxacin, trimethoprim, atenolol, sotalol, furosemide, hydrochlorothiazide, bezafibrate, gemfibrozil, carbamazepine and lorazepam were detected in 100% of the analyzed samples. In contrast, sulfamethazine, sulfamethoxazole, metoprolol, fenofibrate, diazepam and paroxetine were detected in ≤ 50% of samples of both phases, and additionally were measured in very low mean concentrations (< 20 ng L^{-1}), with the exception of the antibiotic sulfamethoxazole. According to the Kruskal-Wallis test, there were no statistically significant differences of the concentrations of the assayed PhACs in the influent wastewater between the two experimental phases, except for carbamazepine, which showed a higher mean concentration in phase I (154 ng L^{-1}) compared to phase II (98 ng L^{-1}). The highest mean influent concentrations were observed for the antibiotic ofloxacin, followed in descending order by furosemide, hydrochlorothiazide, gemfibrozil, clarithromycin, and atenolol (Table 1).

Nine PhACs were detected in 100% of the effluent samples, while the concentrations of 5 compounds (sulfamethazine, metoprolol, fenofibrate, diazepam and paroxetine) were below the limits of detection (Table 2). According to the Kruskal-Wallis test, there were statistically significant differences among the two phases for the concentrations of hydrochlorothiazide, gemfibrozil, and bezafibrate. The highest mean values in the effluent were found for ofloxacin, furosemide, hydrochlorothiazide, and gemfibrozil, while the mean concentrations of the remaining PhACs in the effluent were < 300 ng L^{-1} in both experimental phases.

The concentrations of all the targeted PhACs in both influent and effluent wastewater fell within the ranges previously reported by several authors (Luo et al., 2014; Tran et al., 2018; Verlicchi et al., 2012), except for the antibiotic ofloxacin, which was measured at concentrations exceeding 100,000 ng L^{-1} in both influent and effluent water in two samples of phase II (Wednesday 29/03/2017 and Sunday 02/04/2017). Such variations of the concentration of antibiotics in wastewaters are likely related to outbreaks of infection diseases; in this sense, clear seasonality in the concentrations and influent mass loads of ofloxacin was previously reported by Coutu et al. (2013).

The lowest Predicted No Effect Concentration (PNEC) values described in the recent literature (Orias and Perrodin, 2013; Tran et al., 2018; Verlicchi et al., 2012) for all the tested PhACs are shown in Table 2. The average concentrations of eight of the targeted PhACs in the A^2O effluent (clarithromycin, ofloxacin, sulfamethoxazole, trimethoprim, propranolol, furosemide, gemfibrozil and lorazepam) fell within the ranges previously

reported (Gurke et al., 2015; Kasprzyk-Hordern et al., 2008, 2009; Verlicchi et al., 2012) but were higher than their PNECs, thus posing a risk for aquatic ecosystems (Tran et al., 2018; Verlicchi et al., 2012). In this scenario, implementation of tertiary treatments is desirable to prevent the continuous discharge of these substances into the receiving water bodies.

Table 1. Concentration range, mean concentration, median concentration, relative standard deviation (RSD) and detecti compounds (PhACs) in the influent wastewater samples of the A^2O system in the experimental phases I (n=4) and II (n= used for statistical analyses. The concentrations of those PhACs marked with an asterisk (*) were significantly different bet of detection.

		Influent Water (ng L^{-1})					
Therapeutic groups	**Compounds**	**Phase I (n=4)**					**Phase II (n=5)**
		Range	**Mean**	**Median**	**RSD (%)**	**Freq. (%)**	**Range**
Antibiotic	**Clarithromycin**	88 - 859	447	421	82	100	443- 1100
Antibiotic	**Ofloxacin**	1290 - 5710	2610	1720	80	100	1980- 119000
Antibiotic	**Sulfadiazine**	BLD - 41	16	11	111	75	BLD - 7
Antibiotic	**Sulfamethazine**	BLD	0	0	0	0	BLD - 6
Antibiotic	**Sulfamethoxazole**	BLD - 873	218	0	200	25	BLD - 592
Antibiotic	**Trimethoprim**	48 - 132	92	93	39	100	75- 164
Beta-blocker	**Atenolol**	279 - 1100	635	581	57	100	378- 905
Beta-blocker	**Metoprolol**	BLD	0	0	0	0	BLD- 17
Beta-blocker	**Propranolol**	BLD - 426	136	59	145	75	1 - 87
Beta-blocker	**Sotalol**	25 - 68	41	36	50	100	27- 46
Diuretic	**Furosemide**	2290 - 4720	3215	2925	33	100	1330- 4090
Diuretic	**Hydrochlorothiazide**	2400 - 3630	2790	2565	21	100	1870- 3320
Lipid-regulator	**Bezafibrate**	79 - 181	127	125	41	100	96- 223
Lipid-regulator	**Fenofibrate**	BLD	0	0	0	0	BLD
Lipid-regulator	**Gemfibrozil**	1180 - 2330	1625	1495	33	100	1080- 2160
Psychiatric	**Carbamazepine ***	117 - 248	154	126	41	100	78- 123
Psychiatric	**Diazepam**	BLD - 2	1	0	200	25	BLD - 1
Psychiatric	**Lorazepam**	183 - 364	241	209	34	100	136- 245
Psychiatric	**Paroxetine**	BLD - 11	5	4	120	50	BLD - 11

Table 2. Concentration range, mean concentration, median concentration, relative standard deviation (RSD) and detecti compounds (PhACs) in the effluent wastewater samples of the A^2O system in the experimental phases I (n=4) and II (n= used for statistical analyses. The removal of those PhACs marked with an asterisk (*) was significantly different betwee detection. PNEC: predicted no-effect concentrations of the analyzed PhACs, according to the available literature. When t effluent water was higher that its PNEC value, the latter has been boldfaced.

		Effluent Water (ng L^{-1})						
Therapeutic groups	**Compounds**	**Phase I (n=4)**					**Phase II (n=5)**	
		Range	**Mean**	**Median**	**RSD (%)**	**Freq. (%)**	**Range**	**Mean**
Antibiotic	**Clarithromycin**	117 - 240	168	158	36	100	BLD - 227	55
Antibiotic	**Ofloxacin**	509 - 5410	2075	1190	108	100	1260 - 128000	51636
Antibiotic	**Sulfadiazine**	BLD - 247	64	5	189	50	BLD - 4	1
Antibiotic	**Sulfamethazine**	BLD	0	0	0	0	BLD	0
Antibiotic	**Sulfamethoxazole**	BLD - 372	93	0	200	25	0 - 363	115
Antibiotic	**Trimethoprim**	25 - 131	67	56	72	100	83 - 130	105
Beta-blocker	**Atenolol**	101 - 558	262	194	78	100	58 - 188	110
Beta-blocker	**Metoprolol**	BLD	0	0	0	0	BLD - 8	3
Beta-blocker	**Propranolol**	0 - 458	119	9	190	75	BLD - 572	140
Beta-blocker	**Sotalol**	15 - 50	28	24	54	100	37 - 56	43
Diuretic	**Furosemide**	2170 - 2560	2323	2280	8	100	1340 - 2780	2184
Diuretic	**Hydrochlorothiazide***	755 - 2560	1714	1770	43	100	2110 - 3200	2646
Lipid-regulator	**Bezafibrate***	41 - 104	81	90	34	100	BLD - 54	26
Lipid-regulator	**Fenofibrate**	BLD	0	0	0	0	BLD	0
Lipid-regulator	**Gemfibrozil***	781 - 1270	1085	1145	20	100	87 - 680	377
Psychiatric	**Carbamazepine**	85 - 152	127	136	23	100	99 - 148	114
Psychiatric	**Diazepam**	BLD	0	0	0	0	BLD - 5	1
Psychiatric	**Lorazepam**	185 - 284	216	198	22	100	156 - 245	194
Psychiatric	**Paroxetine**	BLD	0	0	0	0	BLD	0

[1] Verlicchi et al. (2012); [2] Orias and Perrodin (2013); [3] Tran et al. (2018)

Table 3 shows the daily mass loads (ML, mg day^{-1} 1000 inh^{-1}) of the twelve PhACs which were detected in at least 75% of samples of both experimental phases, calculated for the influent and effluent water samples, together with their predicted mass loads (PL) and the corresponding PL/ML influent ratios, calculated as shown in Table S5. Similar influent ML values were found in both experimental phases for each target compound, except for ofloxacin (746 and 13,855 mg day^{-1}1000 inh^{-1} in phase I and II, respectively). The PL/ML ratios were calculated to estimate the accuracy of the PL values, which are considered valid if the PL/ML ratio lies within the 0.5-2.0 range (Ort et al., 2009; Verlicchi et al., 2014). Accordingly, the PL values were acceptable for 6 of the 11 PhACs which were detected in 100% samples of both experimental phases (clarithromycin, trimethoprim, bezafibrate, gemfibrozil, carbamazepine and lorazepam), as well as for propranolol in phase II.

In the A^2O effluent samples, the ML values for clarithromycin, atenolol, propranolol, bezafibrate and gemfibrozil were 3-2-fold lower in phase II compared to phase I (Table 3). Ofloxacin, trimethoprim and hydrochlorothiazide displayed higher ML in phase II compared to phase I, while similar ML values were found for the rest of the targeted PhACs in both phases (Table 3). The variability of the ML values in the effluent was mainly due to the different REs observed in phases I and II, as it will be further discussed in Section 3.2. It has been previously reported that the ML of PhACs in WWTPs influent and effluents are subjected to strong geographic and seasonal variabilities (Jelić et al., 2011; Papageorgiou et al., 2016; Park et al., 2017; Pereira et al., 2015); nevertheless, the data calculated in the present study are comparable with those reported by the aforementioned authors, with the exception of the antibiotic ofloxacin.

Table 3. Daily mass load (ML, mg day^{-1}1000 inh^{-1}), predicted daily mass load (PL, mg day^{-1}1000 inh^{-1}), and PL pharmaceutically active compounds (PhACs) detected in ≥ 75% of the influent and effluent wastewater samples of the I and II.

PhACs (by therapeutic group)	ML Phase I		ML Phase II		PL of A^{2}O System
	Influent	Effluent	Influent	Effluent	Influent
Antibiotics					
Clarithromycin	128	48	224	16	147
Ofloxacin	746	593	13855	14753	1
Trimethoprim	26	19	34	30	19
Beta-blockers					
Atenolol	182	75	175	32	516
Propranolol	39	34	11	12	10
Sotalol	12	8	10	40	26
Diuretics					
Furosemide	919	664	798	624	192
Hydrochlorothiazide	797	490	754	756	185
Lipid-regulators					
Bezafibrate	36	23	40	7	36
Gemfibrozil	464	310	407	108	410
Psychiatric medications					
Carbamazepine	44	36	28	33	29
Lorazepam	69	62	52	55	40

3.2. Removal efficiency of PhACs and links with the environmental/operational variables influencing the A^2O system

Table 4 shows the REs (range, mean, median and RSD) of the target PhACs throughout the experimental phases I and II in the A^2O system. Those compounds with a frequency of detection in samples < 50% and average mean influent concentrations < 20 ng L^{-1} (sulfamethoxazole, sulfamethazine, sulfadiazine, metoprolol, fenofibrate, diazepam and paroxetine) were excluded from the calculations, since conclusions regarding its REs could not be properly drawn.

According to the Kruskal-Wallis test, statistically significant differences of the REs were observed among phases I and II for 5 PhACs (Table 4). Mean REs were higher in phase II for clarithromycin, bezafibrate, and gemfibrozil (93, 80 and 74%, respectively) compared to phase I (30, 33 and 31%, respectively), while the REs of sotalol and hydrochlorothiazide were higher in phase I (28 and 38%, respectively) compared to phase II (-13 and 0%, respectively). The REs of atenolol, furosemide and trimethoprim were no significantly different among the two experimental phases, while very low or negative mean REs were observed for ofloxacin, propranolol, carbamazepine, and lorazepam, in agreement with earlier reports in the literature (Ashfaq et al., 2017; Blair et al., 2015; Gurke et al., 2015; Pereira et al., 2015). Negative REs of PhACs are commonly observed in WWTPs and are attributable to the release of molecules adsorbed/enclosed in suspended particles, or the transformation of conjugate forms into the parent compounds via microbial activity (Ashfaq et al., 2017; Blair et al., 2015; Göbel et al., 2007; Verlicchi et al., 2012).

Hydrochlorothiazide and sotalol displayed better removal rates during phase I, mostly favored by the lower concentrations of MLSS ($\rho < -0.70$, Table S6). On the contrary, the REs of bezafibrate, clarithromycin and gemfibrozil, which were significantly higher in phase II (Table 4), were correlated with higher MLSS concentration in the activated sludge, lower F/M ratios, and higher concentrations of NH_4^+ in the influent water (Table S6). In a previous study in the same A^2O system, it was observed that the REs of acetaminophen, ibuprofen, naproxen, and ketoprofen were also improved under the operating conditions of phase II (Gallardo-Altamirano et al., 2018). Operation at high MLSS concentrations and low F/M ratios has been reported particularly favorable for the improvement of the REs of persistent PhACs (Verlicchi et al., 2012). Increased NH_4^+ loading rates were also previously found correlated to better removal rates of micropollutants in WWTPs, an effect which is attributed to the enhancement of the biological activity of nitrifiers, since it is well established that ammonia oxidizers

Table 4. Removal efficiencies (REs, %) of pharmaceutically active compounds (PhACs) and relative standard deviatio measured in the A^2O system in the experimental phases I (n=4) and II (n=5). The Kruskal Wallis test ($p < 0.05$) was those PhACs marked by an asterisk (*) was significantly different between the two phases. BLD: below the limit of values < 20 ng L^{-1} and a frequency detection < 50% are not shown.

		% RE Water					
Therapeutic groups	**Compounds**	**Phase I**					**Phase II**
		Range	**Mean**	**Median**	**RSD (%)**	**n**	**Range**
Antibiotic	**Clarithromycin ***	-38 - 82	30	37	194	4	74 - 100
Antibiotic	**Ofloxacin**	-319 - 91	-42	31	-448	4	-9 - 45
Antibiotic	**Trimethoprim**	-24 - 56	30	45	121	4	-11 - 29
Beta-blocker	**Atenolol**	-30 - 80	48	71	110	4	69 - 88
Beta-blocker	**Propranolol**	-600 - 100	-90	71	-380	3	-559 - 100
Beta-blocker	**Sotalol ***	-6 - 57	28	31	94	4	-49 - 21
Diuretic	**Furosemide**	-3 - 54	22	19	109	4	-5 - 45
Diuretic	**Hydrochlorothiazide ***	5 - 69	38	39	76	4	-13 - 17
Lipid-regulator	**Bezafibrate ***	0 - 47	33	43	67	4	61 - 100
Lipid-regulator	**Gemfibrozil ***	13 - 45	31	33	43	4	48 - 92
Psychiatric	**Carbamazepine**	-30 - 66	5	-9	887	4	-28 - -2
Psychiatric	**Lorazepam**	-55 - 49	1	6	3446	4	-19 - 2

contribute to the biodegradation of a wide range of trace organic compounds in engineered systems, possibly through cometabolic reactions mediated by the ammonia monooxygenase enzymes (reviewed by Kumwimba and Meng, 2019).

The improvement of the REs of bezafibrate, clarithromycin and gemfibrozil in phase II was concurrent with better removal rates of both organic matter and nutrients, compared to phase I (Table S1). In particular, high positive correlations ($\rho > 0.65$) were displayed among REs of the three PhACs and NRR (Table S6). Interestingly, an association among higher NRR and improved removal of PhACs was also established previously in activated sludges of 10 full-scale WTPPs (Helbling et al., 2012).

3.3. Microbial community structure and diversity in the A^2O system

The biological treatment of wastewater mainly relies on the ability of heterotrophic bacteria and fungi for the biotransformation of organic substances and the removal of nutrients from effluents. At the same time, Archaea fulfil important roles as part of the syntrophic metabolic networks needed to complete the mineralization of organic compounds under anaerobic conditions (Nobu et al., 2015). The diversity of Bacteria, Archaea and Fungi in the A^2O system was surveyed by massive parallel sequencing of the small subunit rRNA genes using the Illumina platform. Sample-size-based and coverage-based R/E sampling curves are shown in Figure S1, and the summary of the average numbers of reads, richness of PHYs (S), Good's coverage and diversity indices calculated for each microbial group is displayed in Table S7.

After the quality filtering of the raw sequencing data, > 120,000 and > 45,000 16S rRNA reads were obtained for Bacteria and Archaea, respectively, for a total of 8 activated sludge samples analyzed. The number of reads per sample ranged between 7864-28740 for Bacteria and 580-14,642 for Archaea, showing differences of the sequencing depth. A total of 1072 Bacteria PHYs and 89 Archaea PHYs were detected at 97% sequences identity, ranging between 481-661 and 23-61 PHYs per sample, respectively. The R/E curves (Figure S1) and the values of Good's coverage (>98%, Table S7) indicated that the achieved sequencing depth sufficed to properly describe the diversity of the bacterial and archaeal communities in the A^2O system. The average values of S, Chao-1, H' and Simpson's indices were very similar in both experimental phases for either Bacteria and Archaea. The indices described bacterial communities displaying high species diversity, while in the case of Archaea the diversity was low-to-medium. Both Bacteria and Archaea communities had a low functional organization, according to the average values of the Simpson's index (Table S7).

The diversity of Fungi in the A^2O system was only investigated by Illumina sequencing in samples of phase I, since this group of microorganisms was not detectable in samples of phase II. A total of 4,922 quality filtered fungal 18S rRNA sequences were obtained from 4 samples. One hundred Fungi PHYs were detected at 97% sequences identity, ranging between 28-74 PHYs per sample. The R/E curves (Figure S1) and the value of Good's coverage (≥ 96 Table S7) indicated enough sequencing depth for the description of fungal diversity in the A^2O samples. The community structure of Fungi was characterized for a low-to-medium species diversity and a low functional organization, according to the average values of S, Chao-1, *H´* and Simpson's indices (Table S7).

3.3.1. Structure and diversity of the Bacteria communities in the A^2O system

The dendrogram in Figure S2 shows the clustering of the A^2O samples (according to the Bray-Curtis coefficient), based on the relative abundance of bacterial PHYs detected by Illumina sequencing. The global similarity among samples in phases I and II was > 40%. Significant differences were also detected between the experimental phases by ANOSIM analysis (R= 0.84).

A total of 1,072 PHYs with a relative abundance > 0.1% in at least one sample were classified in 27 different Phyla. The bacterial community was dominated by *Proteobacteria*, which displayed the highest average relative abundance in the sludge samples of both phases (60.4 and 64.7%; phase I and II, respectively), followed by *Bacteroidetes* (23.1 and 15.04 %) and *Chloroflexi* (7.7 and 11.4 %) (Figure 1A.1) Interestingly, unique Phyla were detected in both experimental phases, although at very low relative abundances. Phylotypes affiliated to Phyla TM6 (0.0065%) and OD1 (0.0020 %) were only detected in phase I, while members of the Phyla GOUTA4 (0.0031%), *Gemmatimonadetes* (0.0036 %) and NKB19 (0.0036 %) were exclusive of phase II. Significant differences of the community composition among the two experimental phases were also detected at the Order and Family levels (Figure 1A.2, A.3).

In order to evaluate which PHYs contributed most to the (dis)similarity between samples of phases I and II, SIMPER analyses were carried out (Table S8). The overall similarity among samples was 61.48% in phase I and 71.09% in phase II. Three PHYs affiliated to the *Comamonadaceae*, *Cytophagaceae*, and *Xanthomonadaceae* (PHYs 962, 406, and 630, respectively) and two PHYs phylogenetic related to *Caldilineaceae* (PHYs 118 and 713) cumulatively contributed > 35% to explain the similarity between phase I samples (Table S8A), while the similarity among phase II samples was explained (> 35%) by PHYs 118, 406 and 962 (Table S8B). SIMPER analysis also showed that PHYs 406 and

962 (Table S8C) were the major contributors to the 47.93% dissimilarity observed between the two experimental phases, cumulative explaining > 18% of the differences.

Proteobacteria, *Bacteroidetes* and *Chloroflexi* have been described as the predominant bacterial groups in aerobic activated sludge of WWTPs, either based on conventional or advanced technologies (reviewed by Ferrera and Sánchez, 2016; Xia et al., 2018). Of these, *Proteobacteria* are most often found as the prevalent group, particularly in municipal WWTPs (Cydzik-Kwiatkowska and Zielińska, 2016). Among these, members of the *Comamonadaceae* are regarded as ubiquitous in activated sludge, where they fulfil a variety of functions such as nitrate reduction (Sadaie et al., 2007), biological phosphorus removal (Ge et al., 2015) and biodegradation of complex substrates (Khan et al., 2002).

3.3.2. Structure and diversity of Archaea communities in the A^2O system

Figure S2 displays the Bray-Curtis based dendrogram showing the (dis)similarities between the A^2O samples of phases I and II, according to the relative abundance of Archaea PHYs detected by Illumina sequencing. Samples of each experimental phase clustered together in separate groups with a similarity < 20%, while the similarity of the samples within each experimental phase was > 40%. ANOSIM analysis also corroborated the significant differences between experimental phases (R=0.188).

Figure 1B (B.1, B.2 and B.3) displays the heatmaps showing the relative abundances of archaeal PHYs in the two experimental phases, at Phylum, Order and Genus level, respectively. *Euryarchaeota* was the dominant Phylum (98.2 and 92.3% average relative abundances, phases I and II, respectively), followed by *Thaumarchaeota* (1.2 and 6.7%) and *Crenarchaeota* (0.1 and 0.4%). Significant differences of the community diversity were also detected among phases I and II at Order and Genus levels (Figure 1B.2 and B.3). *Methanomicrobiales* were prevalent in phase I (average relative abundance 45.4%), while *Methanobacteriales* dominated in phase II (average relative abundance 49.8%). At the Genus level, phase I was dominated by PHYs closely related to *Methanolinea* (*Methanomicrobiales*) (28.9% average relative abundance), while *Methanobrevibacter* and *Methanobacterium* (*Methanobacteriales*) were prevalent in samples of phase II (21.8 and 21.2 % average relative abundance, respectively) (Figure 1B.3).

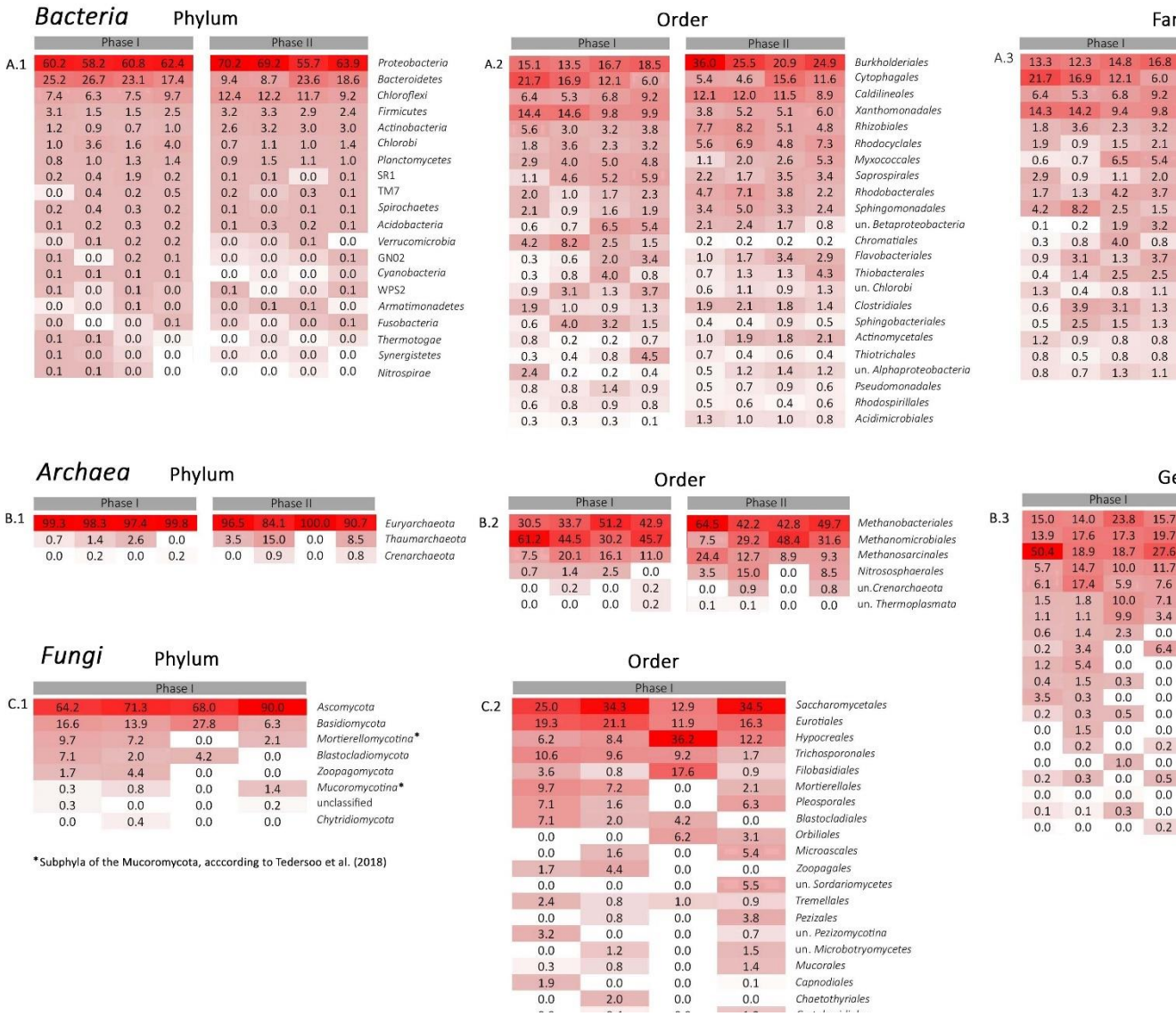

Figure 1. Heatmaps showing the relative abundances of Bacteria (A), Archaea (B) and Fungi (C) detected by Illumina seq under different conditions in experimental phases I and II. A1, B1, C1. Classification at Phylum level. A2. Classification at at Order level. B3. Classification at Genus level. Taxa were excluded from the heatmaps according to the following cut considering both experimental phases: Bacteria: < 0.5, < 0.6, and < 0.9 % for Phylum, Order and Family, respectively; A Genus; Fungi: < 0.4 for Order.

According to SIMPER analysis (Table S9), the global similarity of the Archaea communities was 61.95% in phase I samples and 48.7% in phase II samples. Three PHYs (PHYs 87, 130 and 29, which were closely related to genera *Methanolinea*, *Methanobrevibacter* and *Methanobacterium*, respectively) contributed 60.8% to explain the similarity between samples of phase I, while PHYs 29, 130, 41 and 87 (closely related to *Methanobacterium, Methanobrevibacter*, *Methanomethylovorans* and *Methanolinea*, respectively) contributed 65.06% to the similarity of phase II samples. SIMPER also corroborated the low similarity of the archaeal communities observed between the two experimental phases (47.31% average dissimilarity), with a single PHY (PHY 87, affiliated to *Methanolinea*) contributing a 16.34% explanation to such differences.

Methanobacteriales, *Methanomicrobiales* and *Methanosarcinales* are commonly detected in urban wastewater treatments based on different technologies, including aerated activated sludge systems (Ferrera and Sánchez, 2016). The A^2O technology is characterized by aerobic, anoxic and anaerobic zones in series, subjected to internal recirculation. Under such conditions, usually no significant differences of the composition of the bacterial communities are found among the bioreactors with different redox regimes (Gómez-Silván et al., 2014; Kim et al., 2013; Phan et al., 2016). Methanogenic archaea present in the anaerobic zones under optimal conditions to develop their metabolism enter the aerated zones by recirculation, where their contribution to carbon and nitrogen removal is controversial, although some studies supported their role as enhancers of some biological activities through their symbiotic relationships with Bacteria (Ferrera and Sánchez, 2016).

3.3.3. Structure and diversity of Fungi communities in the A^2O system

The dendrogram in Figure S2 illustrates the clustering of samples (Bray Curtis) according to the relative abundance of the fungal PHYs in the A^2O samples from phase I, showing a low similarity (<20%) among them. PHYs classified in 7 different Phyla/Subphyla were identified. *Ascomycota* was the dominant Phylum (73.4% average relative abundance), followed by *Basidiomycota* (16.2%), *Mortierellomycotina* (*Mucoromycota*, Tedersoo et al., 2018) (4.7%), *Blastocladiomycota* (3.3%), *Zoopagomycota* (1.5%), *Mucoromycotina* (*Mucoromycota*, Tedersoo et al., 2018) (0.63%) and *Chytridiomycota* (0.09%) (Figure 1C.1). At the Order level, 34 different taxa were detected, dominated by *Ascomycota* of the *Saccharomycetales*, *Eurotiales* and *Hypocreales*, and *Basidiomycota* of the *Trichosporonales* and *Filobasidiales* (26.7, 17.2, 15.7, 7.8 and 5.7% average relative abundance, respectively) (Figure 1C.2). SIMPER analysis (Table S10) showed that the global similarity between samples was 39.3%, where

only 5 PHYs affiliated to the *Hypocreales*, *Trichosporonales*, *Saccharomycetales* and *Eurotiales* were enough to explain > 55% of such similarity.

The results presented here revealed a higher diversity and relative abundance of *Ascomycota* compared to *Basidiomycota* in the A^2O system, and in most samples, phylotypes closely related to the *Saccharomycetes* displayed the highest relative abundances within the fungal community (Figure 1C), in agreement with the knowledge of the diversity of fungi in WWTPs (Maza-Márquez et al., 2016). *Ascomycota* are an extremely diverse group of fungi, accounting for > 65% of these eukaryotic microorganisms in both terrestrial and aquatic environments (Harms et al., 2011). Several species have been described able to biotransform PhACs, including AIAPs, antibiotics, or β-blockers (Olicón-Hernández et al., 2017).

3.4. Linking the relative abundance of Bacteria and Archaea to the environmental/operational variables of the A^2O system

In Figure 2 (A, B), the activated sludge samples retrieved from the A^2O system were ordinated by MDS on the basis of the relative abundance of the populations of Bacteria and Archaea identified by Illumina-sequencing. Fungi were not included in these analyses, since they were only detected in samples of phase I. The vectors on the plots representing the abiotic variables displayed two clear trends throughout the ordinations, in agreement with the different operating conditions characterizing each of the two experimental phases (higher average OT and F/M in phase I, and higher concentrations of COD and BOD_5 in the influent water, higher biomass accumulation (MLSS) and sludge volumetric index (SVI) in phase II) and the increased removal efficiencies of organic matter and nutrients (ORR, NRR, PRR) registered during phase II. These changes in the operational and environmental parameters were strongly related to the shifts of the structure of the microbial communities in the A^2O system (Figure 2), as reported previously for WWTPs by several studies (Ju and Zhang, 2015; Kim et al., 2013; Reboleiro-Rivas et al., 2016).

According to BIO-ENV analysis, among all the tested variables, MLSS, SRT, COD, BOD_5 and NH_4^+ were those contributing the highest level of explanation (89.1%) to the ordination of samples based on the bacterial community structure (Figure 2A). The Pearson product-moment correlation coefficients among the vectors in Figure 2A (Table S11) showed that MLSS, COD, BOD_5 and NH_4^+ were positively correlated ($r \geq 0.70$) to the relative abundance of families of the *Alphaproteobacteria* (*Hyphomicrobiaceae*, *Rhodobacteraceae*, *Sphingomonadaceae*, unclassified *Rhizobiales*,

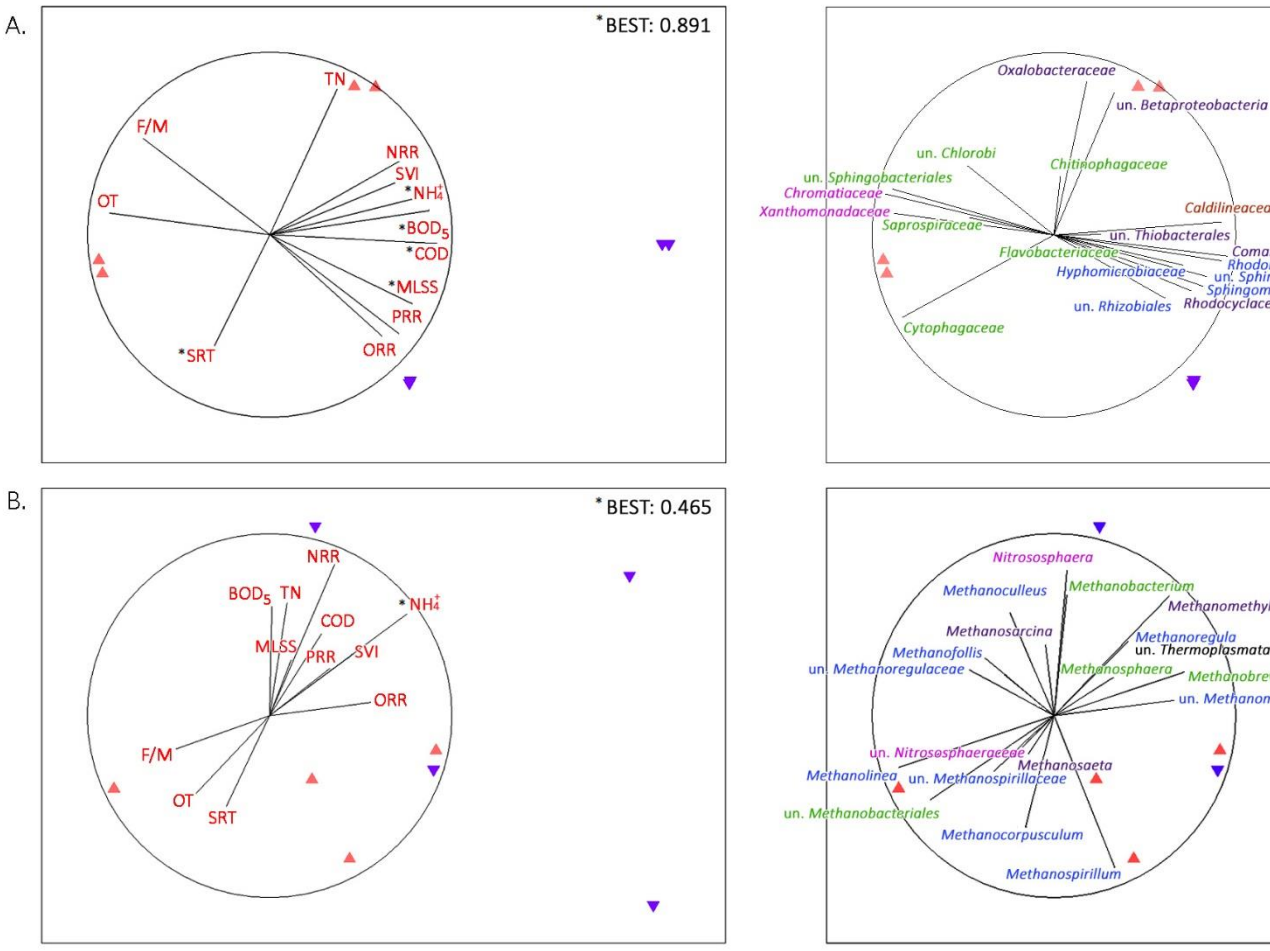

Figure 2. Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the samples retrieved experimental conditions I and II, according to the relative similarity of Bacteria (A) and Archaea (B) analyzed by Illum MDS plots represent the direction throughout the ordination of the relative abundances of Bacteria, Archaea and Fun MDS plots represent the abiotic variables measured in the A^2O system. BOD_5: biological oxygen demand at 5 days; CO to-microorganism ratio; MLSS: mixed suspended solids; OT: operational temperature; TN: Total N; SRT: sludge reten The variables which best explained the distributions of the biotic data according to BIO-ENV analysis are marked which >0.2 are shown.

and unclassified *Sphingomonadales*), *Betaproteobacteria* (*Comamonadaceae*, *Rhodocyclaceae*, and unclassified *Thiobacterales*) and *Chloroflexi* (*Caldilineaceae*). In contrast, the relative abundances of PHYs related to the *Bacteroidetes*/*Chlorobi* (*Cytophagaceae*, *Saprospiraceae*, Unclassified *Chlorobi*, and Unclassified Sphingobacteriales) and *Gammaproteobacteria* (*Chromaticeae* and *Xanthomonadaceae*) showed opposed trends throughout the samples' ordination.

Some of the responses of the bacterial groups to the changes of the operational parameters in the A^2O were in agreement with those observed in previous studies. The increase of MLSS in phase II contributed to higher relative abundances of *Caldilineaceae* and *Chloroflexi* populations (Figure 2A. and Table S11A), accordingly to the previous observations in a moving-bed membrane bioreactor (MBMBR) treating municipal sewage (Reboleiro-Rivas et al., 2016). Muszyński et al. (2015) analyzed the bacterial diversity in an A^2O system by FISH probing, reporting the positive correlation of the relative abundances of both *Alpha-* and *Betaproteobacteria* with COD and total N of the influent wastewater and their negative correlation with OT, while *Bacteroidetes* of the *Saprospiraceae* followed the opposite trend.

Regarding the relative abundances of Archaea populations at the Genus level, BIO-ENV analysis detected that NH_4^+ concentration in the activated sludge was the parameter contributing the best explanation to the ordination of samples (46.5%). These results indicate an effect of NH_4^+ concentration on the succession of both hydrogenotrophic and methylotrophic/acetoclastic methanogens. Strong positive correlations of this abiotic factor ($r > 0.65$) were observed with the relative abundances of most PHYs of the *Methanobacteriales* identified at the genus level, while negative or negligible correlations were observed for the genera of the *Methanomicrobiales* (with the exception of *Methanoregula*). Higher NH_4^+ concentrations also favored the dominance of *Methanomethylovorans* and *Methanosarcina* versus *Methanosaeta* (Figure 2B and Table S11B).

Ammonia concentration is regarded as an important parameter for the shaping of the methanogenic communities. NH_4^+ is required as the major N source for most methanogens (Garcia et al., 2000), but at the same time ammonia ions and free ammonia can inhibit methanogenesis (Yenigün and Demirel, 2013). The effect of ammonia on population dynamics and the activity of methanogens has been thoroughly described in anaerobic digesters at much higher average concentrations (usually >1 g L^{-1}) than those measured in the A^2O system investigated here; however, very similar patterns of community succession in response to ammonia were observed. Previous studies concluded that hydrogenotrophic

methanogens are more tolerant to ammonia levels than acetotrophic methanogens, and the obligate acetoclastic *Methanosaeta* are the most sensitive to ammonia (Karakashev et al., 2005; Yenigün and Demirel, 2013). In this regard, *Methanosaetaceae* dominated in sewage sludge digesters operating at low ammonia levels (0.03-0.3 g $N\text{-}NH_4^+$ L^{-1}), while *Methanosarcinaceae* were prevalent in cattle manure digesters with concentrations of $N\text{-}NH_4^+$ >1 g L^{-1} (Karakashev et al., 2005). Among the hydrogenotrophic methanogens, *Methanobacteriales* increased their abundance versus *Methanomicrobiales* at higher NH_4^+ loading rates in a laboratory-scale two-stage anaerobic digester operated in the range of 0.09-1.16 g NH_4^+ L^{-1} day (Kim et al., 2014).

Nitrososphaera is an ammonia oxidizing archaea (AOA) described in either water or soil environments (Ma et al., 2018; Mosier et al., 2012; Park et al., 2012), and is the AOA most often identified in WWTPs (Limpiyakorn et al., 2013). In that respect, the average relative abundance of *Nitrososphaera* increased from 1.05 % in phase I to 6.74 % in phase II, and strong positive correlations ($r > 0.65$) were found between the abundance of these AOA and NH_4^+, TN and NRR (Table S11B). Ammonia-oxidizing bacteria (AOB) have been classically considered the key organisms responsible of nitrification in WWTPs (Yin et al., 2018); however, in the last decades it has been established that AOA of the *Thaumarchaeota* often coexist with AOB in these engineered systems (Limpiyakorn et al., 2013), within an ample range of ammonia concentrations (Yin et al., 2018), and their effective contribution to ammonia oxidation has been recently demonstrated in nitrifying reactors (Roy et al., 2017; Srithep et al., 2018). The results of the present study show that the relative abundance of *Nitrososphaera*-related phylotypes among Archaea was favored by increasing concentrations of ammonia in the A^2O influent, and correlated concomitantly with improved NRR, supporting the relevance of their role in ammonia oxidation. Similar correlation trends were previously found for *Nitrososphaera*-related AOA by Gao et al. (2014).

3.5. Linking population dynamics of Bacteria and Archaea to the REs of PhACs in the A^2O system

MDS and BIO-ENV analyses were used in search of links among the observed changes of the bacterial and archaeal communities and the REs of PhAC in the A^2O system. Fungi were not included in these analyses, since they were only detected in samples of phase I. Besides the data of 7 of the PhACs described in this study (Table 4), the REs of 4 AIAPs (acetaminophen, ibuprofen, naproxen, and ketoprofen) which were previously

reported in the same set of samples (Gallardo-Altamirano et al., 2018) were also included. The samples' ordination plots are shown in Figure 3.

BIO-ENV analysis revealed strong correlations between the removal of PhACs of different pharmaceutical families (AIAPs, antibiotic, β-blockers, diuretics and lipid-regulator) and the relative abundances of particular bacterial and archaeal populations (Figure 3 and Table S12). Overall, the trends of the vectors on the plot reflects that the REs of most of the analyzed PhACs were higher in experimental phase II, with the exception of hydrochlorothiazide. BIO-ENV analyses showed that the REs of the PhACs were positively correlated with the increased relative abundance of a core of different bacterial families during phase II (Figure 3A). Higher relative abundances of *Rhodobacteraceae* and *Sphingomonadaceae* (*Alphaproteobacteria*) were strongly correlated ($r > 0.75$) with increased REs of clarithromycin, furosemide, bezafibrate and gemfibrozil, while *Betaproteobacteria* (*Comamonadaceae, Rhodocyclaceae,* unclassified *Betaproteobacteria* and unclassified *Thiobacterales*) and *Chloroflexi* (*Caldilineaceae*) correlated positively ($r = 0.75$-1.0) with the REs of up to seven PhACs belonging to different therapeutic groups: AIAPs (ibuprofen and naproxen), antibiotics (clarithromycin), diuretics (furosemide), β-blockers (atenolol) and lipid-regulators (bezafibrate, gemfibrozil) (Table S12A). The relative abundances of phylotypes of the *Chitinophagaceae* and *Flavobacteriaceae* (*Bacteroidetes*/*Chlorobi*) were also correlated to higher REs of ibuprofen and naproxen, while *Gammaprotobacteria* and *Cytophagaceae* abundances displayed a very high positive correlation with the REs of hydrochlorothiazide ($r > 0.9$), the only PhAC which was removed with a significantly higher efficiency under the operating conditions of phase I (Table S12A). Finally, the REs of acetaminophen were strongly and positively correlated ($r > 0.9$) to the relative abundance of unclassified *Myxococcales* (*Deltaproteobacteria*) and some groups of the *Bacteroidetes*/*Chlorobi* (Figure 3, Table S12A).

Regarding the archaeal population, MDS and BIO-ENV detected strong correlations ($r \geq 0.60$) between the REs of different AIAPs (acetaminophen, ibuprofen, ketoprofen and naproxen), antibiotics (clarithromycin), β-blockers (atenolol) and lipid-regulators (bezafibrate and gemfibrozil) and the relative abundances of *Methanobrevibacter, Methanoregula, Methanosaeta, Methanosphaera,* and unclassified *Methanomicrobiales.* The relative abundance of *Methanomethylovorans* was positively correlated ($r \geq 0.60$) with the REs of ibuprofen, naproxen, clarithromycin, atenolol, furosemide, bezafibrate and gemfibrozil (Table S12B).

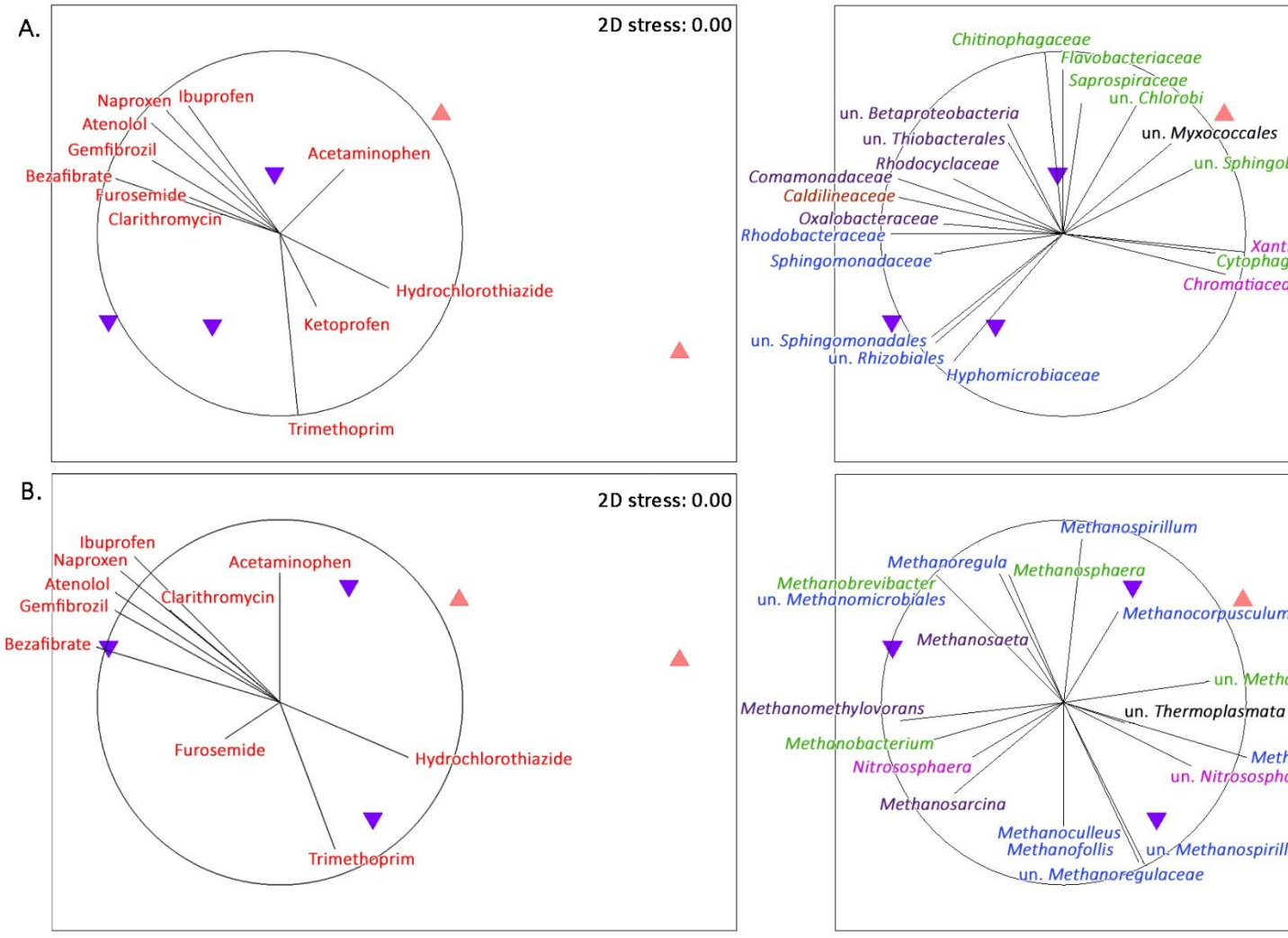

Figure 3. A Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the activated sludge s experimental phases I and II, according to the relative similarity of Bacteria (A) and Archaea(B) communities analyzed column, the MDS plots represent the direction throughout the ordination of the relative abundances of the Bacteria and column, MDS plots represent removal rates (REs%) of acetaminophen, ibuprofen, ketoprofen, naproxen, clarithromyci hydrochlorothiazide, bezafibrate and gemfibrozil. Only vectors with length >0.2 are shown.

The performance of biological wastewater treatment systems for PhACs removal has been thoroughly investigated in systems based on different technologies, but only a few studies provided significant links among their REs and the occurrence/abundance of particular microbial groups, most of which were conducted in laboratory-scale systems using synthetic wastewater spiked with fixed amounts of PhACs, and often using concentrations well over those occurring in real wastewaters. In spite of this differences, the results presented here are consistent with those of previous work. *Alphaproteobacteria* and *Betaproteobacteria* have been often linked to the biological removal of PhACs in WWTPs based on different technologies. The relative abundances of populations classified within the *Sphingomonadaceae* were earlier found positively correlated with the REs of the antibiotic trimethoprim in an autotrophic nitrogen removal CANON bioreactor (Rodriguez-Sanchez et al., 2017), and two genera of the *Sphingomonadaceae* (*Sphingorhabdus* and *Novosphingobium*) were pointed out as indicators of conditions favorable for the removal of bezafibrate in both a full-scale and a pilot-scale WWTP (Wolff et al., 2018). The enrichment in the activated sludge communities of phylotypes related to several genera of the *Comamonadaceae* and *Rhodocyclaceae* was previously observed to occur concomitantly with the removal of bezafribrate and clarithromicyn (Wolff et al., 2018), ibuprofen (Li et al., 2016), and trimethoprim (Rodriguez-Sanchez et al., 2017). Isolates taxonomically classified within the *Comamonadaceae* and *Sphingomonadaceae* are also commonly reported able to metabolize/cometabolize different kinds of PhACs under laboratory conditions, either in pure cultures or as part of consortia (Fortunato et al., 2016; Wang and Wang, 2016).

Regarding the links found among the REs of PhACs and other bacterial groups, an increase in the relative abundance of *Flavobacteriaceae* (*Bacteroidetes*) was observed in a pilot-scale MBR fed with synthetic hospital wastewater following the addition of 10 μg L^{-1} ibuprofen (Tiwari et al., 2019), and populations of this family have been as well associated with the metabolic or cometabolic degradation of ibuprofen in constructed wetlands (Li et al., 2016). Enrichment of *Bacteroidetes* and *Chloroflexi* in an aerated SBR fed with synthetic wastewater amended with a mixture of diclofenac, ibuprofen and naproxen (5 μg L^{-1} each) was reported by Jiang et al. (2017); however, these authors found that the relative abundance of *Proteobacteria* was at the same time lowered down under the conditions of their study, contrary to the results presented here. Phan et al. (2016) assayed an MBR operated under different sets of conditions for the removal of 30 trace organic contaminants (including gemfibrozil, ibuprofen, ketoprofen, and naproxen) at a concentration of 5 μg L^{-1}, reporting that *Proteobacteria* increased their relative abundance in the presence of the

trace organic contaminants, while *Bacteroidetes* (*Cytophagaceae*) mostly thrived in their absence, and concluding that *Betaproteobacteria*, *Caldilineaceae* and *Myxococcales* were more abundant among the activated sludge community under the operating conditions allowing the effective removal of the micropollutants.

The role of Archaea in PhACs removal in WWTPs has been even more scarcely investigated. Methanogenic archaea are regarded a key component in the biodegradation of pollutants such as hydrocarbons in soil and water, where they thermodynamically favor the degradative processes by acting as a sink of the terminal products H_2 and acetate (Krzmarzick et al., 2018). The acetoclastic genus *Methanosaeta* significantly increased its abundance in diesel-contaminated versus non-contaminated soils (Krzmarzick et al., 2018) and also dominated the community in a WWTP treating industrial wastewater containing azo-dyes (Plumb et al., 2001). *Methanomethylovorans*, a obligately methylotrophic methanogen able to use methanol, methanethiol, methylamines and dimethyl sulfide as substrates (Lomans et al., 1999), has been described as the dominant methanogenic archaea in WWTPs treating high concentrations of antibiotics, pointing at their implication together with sulfate reducing prokaryotes in the latter steps of biodegradation of these compounds under anaerobic conditions (Deng et al., 2012). A similar role of *Methanomethylovorans* in the anaerobic degradation of dyes with sulfonate groups in industrial wastewater was earlier proposed (Plumb et al., 2001).

The relative abundance of *Nitrososphaera*-related phylotypes within the archaeal community of the A^2O displayed positive correlations with the REs of furosemide, bezafibrate and gemfibrozil (Figure 3, Table S12). It was previously shown that the removal of these three PhACs, among others, was significantly hampered following chemical inhibition of ammonia monooxygenase activity in bench-scale MBRs and anthracite-packed biofilters (Maeng et al., 2013; Rattier et al., 2014). In the literature, the involvement of nitrifiers in the removal of trace organic contaminants from wastewater has been mostly attributed to AOB (Kumwimba and Meng, 2019); however, in a survey of activated sludge samples from 10 full-scale WWTPs, Helbling et al. (2012) concluded that both ammonia oxidation and the removal of PhACs were correlated to the abundance of archaeal, rather than bacterial, *amoA* transcripts.

4. Conclusion

The findings presented here provide new insights on the correlations among plant operation parameters and the assemblage of the activated sludge microbial communities, regarding how these interlinkages influence the removal of PhACs in WWTPs. Robust

links were revealed between improved REs of several of the analyzed compounds (acetaminophen, ibuprofen, ketoprofen, naproxen, clarithromycin, trimethoprim, atenolol, furosemide, hydrochlorothiazide, bezafibrate, and gemfibrozil) and the shifts of the relative abundances of different families of *Bacteria* (*Rhodobacteraceae*, *Sphingomonadaceae*, *Comamonadaceae*, *Rhodocyclaceae*, *Caldilineaceae*, *Chitinophagaceae* and *Flavobacteriaceae*) and genera of *Archaea* (*Methanobrevibacter*, *Methanoregula*, *Methanosaeta*, *Methanosphaera*, *Methanomethylovorans* and *Nitrososphaera*). Remarkably, the REs of clarithromycin, bezafibrate and gemfibrozil were improved over twofold by the combination of an average MLSS concentration of *ca.* 5 g L^{-1} with a F/M ratio of 0.21 kg BOD_5 kg MLVSS $^{-1}$ d^{-1}. These operational parameters are easy-to-control and their adjustment feasible in seek for an improvement of the removal of PhACs in WWTPs.

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Supplementary material for Chapter 2:

"Linking microbial diversity and population dynamics to the removal efficiency of pharmaceutically active compounds (PhACs) in an anaerobic/anoxic/aerobic (A^2O) system".

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Figure S1. Sample-size-based and coverage-based rarefaction and extrapolation (R/E) phylotype accumulation
intervals) calculated for Bacteria, Archaea and Fungi in the activated sludge samples of the A^2O system through
and II. Solid lines are estimates of richness via interpolation, while dashed lines are extrapolations of richness fo
sample sizes.

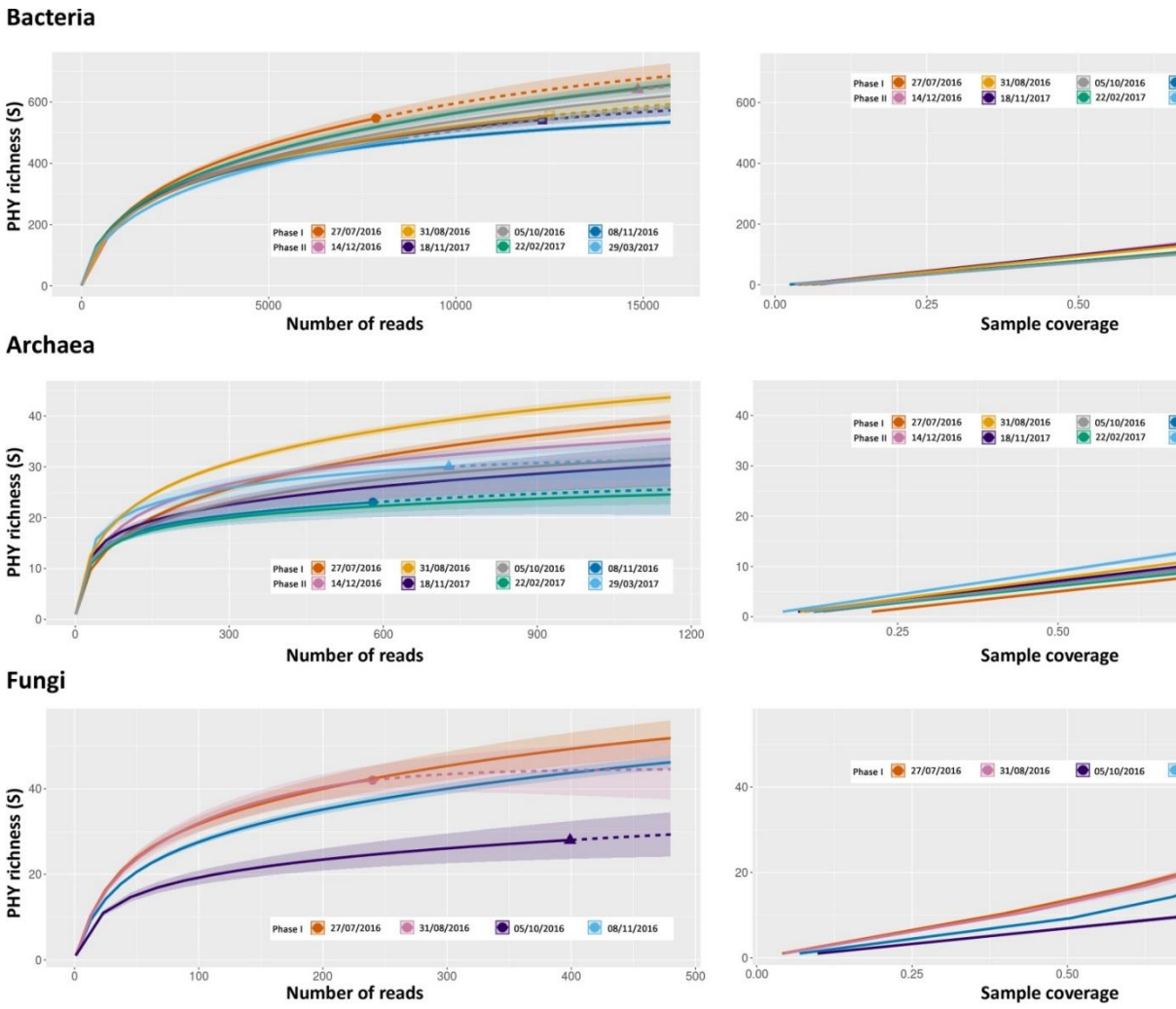


Figure S2. Dendrograms showing the similarities among the Bacteria (A), Archaea (B) and Fungi (C) communities in the activated sludge samples of the A^2O system throughout the experimental phases I and II, based on the analysis of the small-subunit rRNA sequences. Clustering was generated by the UPGMA algorithm using the Bray-Curtis distance similarity matrix generated from the relative abundances of the phylotypes identified by Illumina-sequencing. Lines in red mark the non-significantly different branches, according to SIMPROF analysis ($p>0.05$).

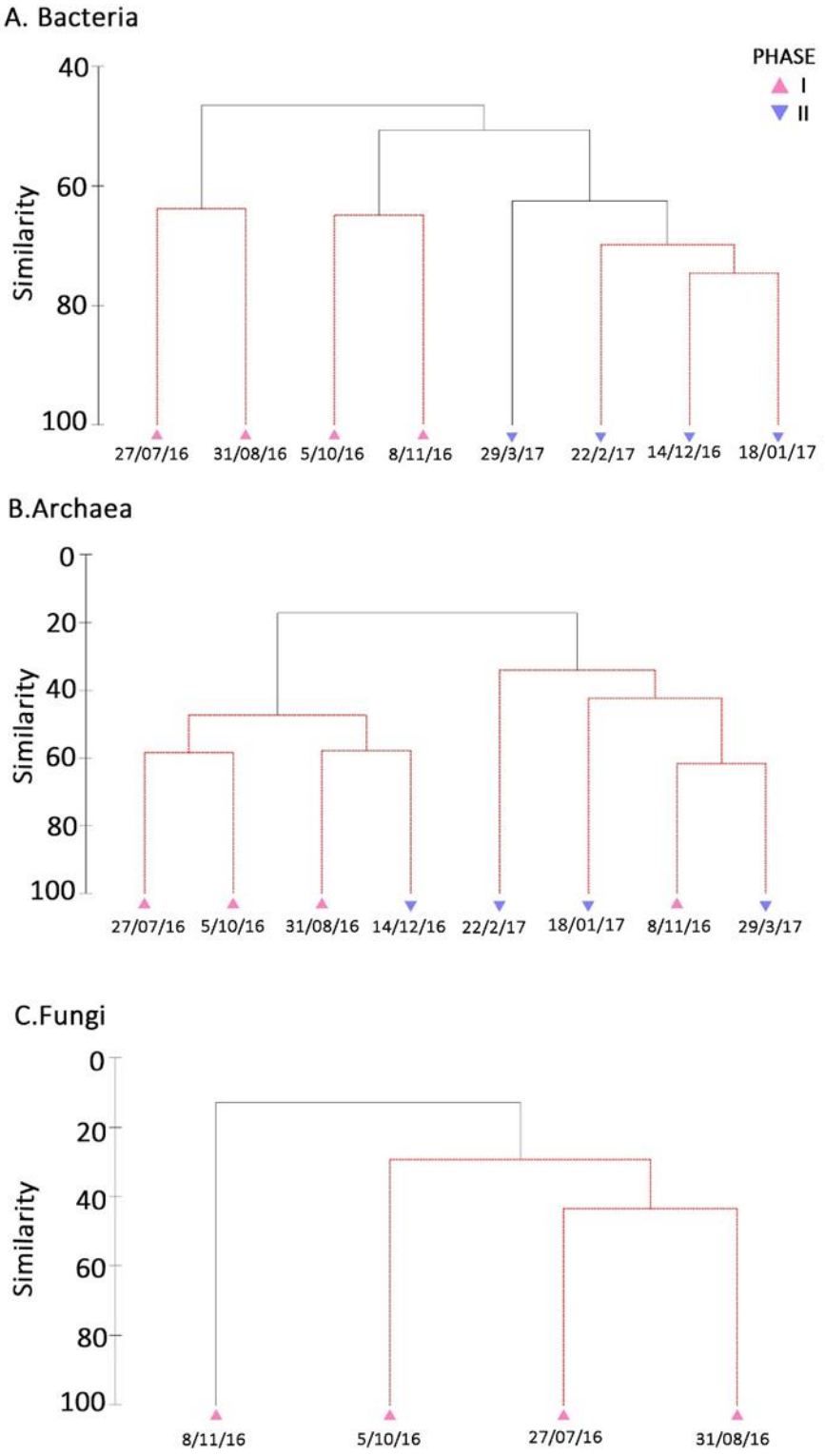


Table S1. Average values (± standard deviation) for operational parameters of the A^2O system during phase I and phase
significantly different between phases, according to the Kruskal-Wallis test ($p<0.05$). **B**. Average ± standard deviations of
in influent water samples during phases I and II in the A^2O system. Data marked with an asterisk (*) are significantly diff
Kruskal-Wallis test ($p<0.05$)

A.

	Influent ($L\ h^{-1}$)	**HRT (h)**	**RAS (%)**	**MLR (%)**	**SRT (d)**	**SVI ($mL\ g^{-1}$)**	**DO set po ($mg\ L^{-1}$)**
Phase I	237 ± 23	7.0 ± 0.7	58 ± 6*	170 ± 39	12.4 ± 4.4	84 ± 24*	0.5 - 1
Phase II	243 ± 32	6.8 ± 0.9	74 ± 10*	209 ± 27	13.1 ± 2.8	106 ± 22*	0.5 - 1

	MLSS ($mg\ L^{-1}$)	**MLVSS ($mg\ L^{-1}$)**	**OT (ºC)**	**OLR ($kg\ BOD_5\ m^{-3}\ d^{-1}$)**	**ORR (%)**	**NLR ($Kg\ TN\ m^{-3}\ d^{-1}$)**	**NRR (%)**
Phase I	2663 ± 434*	2096 ± 98*	24.3 ± 2.9*	0.625 ± 0.173*	91.6 ± 4.0*	0.181 ± 0.020	56.6 ± 1
Phase II	5079 ± 529*	3992 ± 147*	17.2 ± 2.5*	0.851 ± 0.272*	95.6 ± 2.0*	0.194 ± 0.023	67.5 ± 3

B.

	COD ($mgO_2\ L^{-1}$)	**BOD_5 ($mgO_2\ L^{-1}$)**	**TSS ($mg\ L^{-1}$)**	**TN ($mg\ L^{-1}$)**	**$N\text{-}NH_4^+$ ($mg\ L^{-1}$)**	**$N\text{-}NO_3^-$ ($mg\ L^{-1}$)**	**T ($mg\ L^{-1}$)**
Phase I	338 ± 57 *	189 ± 43 *	104 ± 30 *	53.4 ± 10.5	40.2 ± 7.5	0.3 ± 0.3	4
Phase II	424 ± 103 *	234 ± 78 *	145 ± 47 *	51.2 ± 10.6	41.5 ± 12.5	0.2 ± 0.4	5

HRT: hydraulic retention time; RAS: return activate sludge rate; MLR: mixed liquor recycle ratio; SRT: sludge retention t
dissolved oxygen; F/M: food-to-microorganisms ratio; MLSS: mixed liquor suspended solids; MLVSS: mixed liquor volat
temperature; Organic loading rate (OLR), nitrogen loading rate (NLR) and phosphorous loading rate (PLR) with the corres
(ORR), Nitrogen Removal Rate (NRR) and Phosphorous Removal Rate (PRR), COD: chemical oxygen demand; BOD_5: b
TSS: total suspended solids, TN: total nitrogen; TP: total phosphorous. HRT, RAS, MLR, SRT, SVI, F/M, OLR, NLP and
Tchobanoglous et al. (2003). Data from Gallardo-Altamirano et al., 2018.

Table S2. List of pharmaceutical standards by therapeutic group (A) and isotopically-labelled compounds used as surrogates (B). All pharmaceutical standards were of high purity grade (>98%).

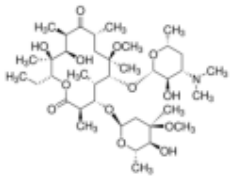
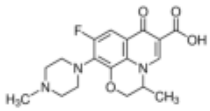
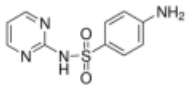
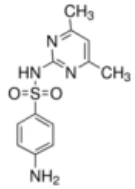
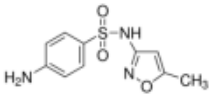
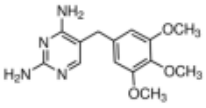
A.

Pharmaceutical compounds	Purchased from
Antibiotics	
Clarithromycin	Cerilliant (Texas, USA)
Ofloxacin	Sigma-Aldric (Steinheim, Germany)
Sulfadiazine	Sigma-Aldrich (Steinheim, Germany)
Sulfamethazine	Sigma-Aldrich (Steinheim, Germany)
Sulfamethoxazole	Sigma-Aldrich (Steinheim, Germany)
Trimethoprim	Sigma-Aldrich (Steinheim, Germany)
Antihypertensives/Diuretics	
Furosemide	Sigma-Aldrich (Steinheim, Germany)
Hydrochlorothiazide	Sigma-Aldrich (Steinheim, Germany)
β-Blockers	
Atenolol	LGC Promochem (London, UK)
Metoprolol	Sigma-Aldrich (Steinheim, Germany)
Propranolol	Sigma-Aldrich (Steinheim, Germany)
Sotalol (hydrochloride)	Sigma-Aldrich (Steinheim, Germany)
Lipid regulators	
Bezafibrate	Sigma-Aldrich (Steinheim, Germany)
Fenofibrate	Jescuder (Rubí, Spain)
Gemfibrozil	Sigma-Aldrich (Steinheim, Germany)
Psychiatric medications	
Carbamazepine	Sigma-Aldrich (Steinheim, Germany)
Diazepam	Cerilliant (Texas, USA)
Lorazepam	Cerilliant (Texas, USA)
Paroxetine	LGC Promochem (London, UK)

B.

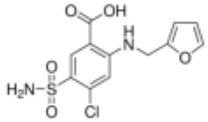
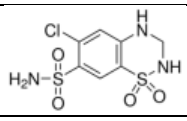
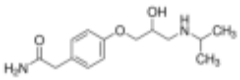
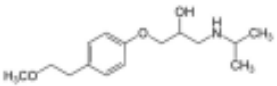
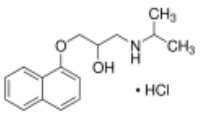
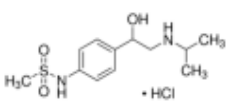
Isotopically labeled compounds	Purchased from
Atenolol-d7	CDN Isotopes (Quebec, Canada)
Bezafibrate-d4	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Carbamazepine-d10	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Diazepam-d5	Cerilliant (Texas, USA)
Fenofibrate-d6	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Furosemide-d5	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Gemfibrozil-d6	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Hydrochlorothiazide-d2	CDN Isotopes (Quebec, Canada)
Rac-Metoprolol-d7	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Paroxetine-d4	LGC Promochem (London, UK)
Propranolol-d7	Cerilliant (Texas, USA)
Sotalol-d6 (hydrochloride)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Sulfadiazine-d4	Sigma-Aldrich (Steinheim, Germany)
Sulfamethazine-d4	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Sulfamethoxazole-d4	Santa Cruz Biotechnology (Santa Cruz, CA, USA)

Table S3. Target compounds with surrogates.

Compound	Structure	CAS Number	Molecular formula	Molecular weight	Precursor ion (*m/z*)	MRM (m/z) Quantification
Antibiotics						
Clarithromycin		81103-11-9	$C_{38}H_{69}NO_{13}$	747.953	748.0 $[M+H]^+$	158
Ofloxacin		82419-36-1	$C_{18}H_{20}FN_3O_4$	361.367	361.4 $[M+H]^+$	318.4
Sulfadiazine		68-35-9	$C_{10}H_{10}N_4O_2S$	250.277	251.0 $[M+H]^+$	156
Sulfamethazine		57-68-1	$C_{12}H_{14}N_4O_2S$	278.330	279.0 $[M+H]^+$	124
Sulfamethoxazole		723-46-6	$C_{10}H_{11}N_3O_3S$	253.278	254.0 $[M+H]^+$	156
Trimethoprim		738-70-5	$C_{14}H_{18}N_4O_3$	290.318	291.0 $[M+H]^+$	230

[a]Internal Standard differs from original method.

Table S3 – (continues)

Compound	Structure	CAS Number	Molecular formula	Molecular weight	Precursor ion (*m/z*)	MRM (m/z) Quantification
Antihypertensives/Diuretics						
Furosemide		54-31-9	$C_{12}H_{11}ClN_2O_5S$	330.744	329.0 $[M-H]^-$	284.6
Hydrochlorothiazide		58-93-5	$C_7H_8ClN_3O_4S_2$	297.739	296.0 $[M-H]^-$	78
β-Blockers						
Atenolol		29122-68-7	$C_{14}H_{22}N_2O_3$	266.336	267.0 $[M+H]^+$	145
Metoprolol		37350-58-6	$C_{15}H_{25}NO_3$	267.364	268.0 $[M+H]^+$	133
Propranolol		525-66-6	$C_{16}H_{21}NO_2$	259.343	260.0 $[M+H]^+$	183
Sotalol		3930-20-9	$C_{12}H_{20}N_2O_3S$	272.364	273.0 $[M+H]^+$	213

[a]Internal Standard differs from original method.

Table S3– (continues)

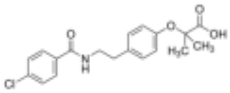
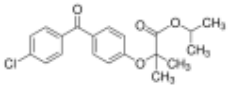
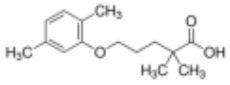
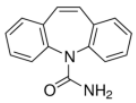
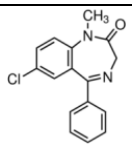
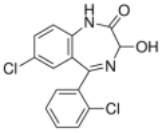
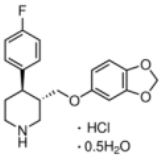
Compound	Structure	CAS Number	Molecular formula	Molecular weight	Precursor ion (*m/z*)	MRM (m/z) Quantification
Lipid regulators						
Bezafibrate		41859-67-0	$C_{19}H_{20}ClNO_4$	361.819	360.0 $[M-H]^-$	274
Fenofibrate		49562-28-9	$C_{20}H_{21}ClO_4$	360.831	361 $[M+H]^+$	139
Gemfibrozil		25812-30-0	$C_{15}H_{22}O_3$	250.333	249.0 $[M-H]^-$	120.8
Psychiatric medications						
Carbamazepine		298-46-4	$C_{15}H_{12}N_2O$	236.269	237.0 $[M+H]^+$	194
Diazepam		439-14-5	$C_{16}H_{13}ClN_2O$	284.740	285 $[M+H]^+$	193
Lorazepam		846-49-1	$C_{15}H_{10}Cl_2N_2O_2$	321.158	323 $[M+H]^+$	277
Paroxetine		61869-08-7	$C_{19}H_{20}FNO_3$	329.365	330.0 $[M+H]^+$	192

Table S4. Compounds and their optimized QqLIT-MS/MS parameters by SRM negative and positive ionization mode and

Compounds	**Precursor ion (m/z)**	**SRM 1**	**DP-CE-CXP**	**SRM 2**	**DP-CE-CXP**	**Rt (min)**	**SR /SR**
Atenolol	267.0 $[M+H]^+$	145	60-35-8	190	60-35-14	3.8	3.1
Atenolol-d7 (S)	274.0 $[M+H]^+$	145	71-37-12	-	-	3.8	-
Bezafibrate	360.0 $[M-H]^-$	274	70-26-1	154	70-38-5	8.2	2.3
Bezafibrate-d4 (S)	364.0 $[M-H]^-$	278	76-26-1	-	-	8.2	-
Carbamazepine	237.0 $[M+H]^+$	194	76-29-19	-	-	10.4	-
Carbamazepine-d10 (S)	247.0 $[M+H]^+$	204	71-31-16	-	-	10.3	-
Clarithromycin	748.0 $[M+H]^+$	158	61-40-12	591	61-35-12	9.8	33.
Diazepam	285 $[M+H]^+$	193	91-45-8	154	91-50-15	13.4	1.5
Diazepam-d5 (S)	290 $[M+H]^+$	198	86-43-16	-	-	13.4	-
Fenofibrate	361 $[M+H]^+$	139	76-43-10	-	-	12.8	-
Fenofibrate-d6 (S)	366.8 $[M+H]^+$	144.9	56-43-8	-	-	12.8	-
Furosemide	329.0 $[M-H]^-$	284.6	65-22-19	205	65-32-11	6.1	1
Furosemide-d5 (S)	334.0 $[M-H]^-$	289.8	50-24-13	-	-	6	-
Gemfibrozil	249.0 $[M-H]^-$	120.8	85-20-7	127.2	85-14-5	15.6	27.
Gemfibrozil-d6 (S)	255.0 $[M-H]^-$	126.8	85-20-7	-	-	13.3	-

Table S4 – (continues)

Compounds	Precursor ion (m/z)	SRM 1	DP-CE-CXP	SRM 2	DP-CE-CXP	Rt (min)	SRM /SR
Hydrochlorothiazide	296.0 $[M-H]^-$	78	90-46-1	268	90-28-17	5.9	107
Hydrochlorothiazide-d2 (S)	298.0 $[M-H]^-$	269.9	60-28-11	-	-	5.9	-
Lorazepam	323 $[M+H]^+$	277	66-45-8	229	66-45-8	11.6	2.6
Metoprolol	268.0 $[M+H]^+$	133	60-35-8	121	60-35-10	6.5	1.4
Metoprolol-d7 (S)	275.2 $[M+H]^+$	123.1	76-25-10	-	-	6.5	-
Ofloxacin	361.4 $[M+H]^+$	318.4	86-29-24	-	-	5.8	-
Paroxetine	330.0 $[M+H]^+$	192	76-31-2	123	76-45-2	9.7	1.4
Paroxetine-d4 (S)	334.0 $[M+H]^+$	196	76-31-2	-	-	9.6	-
Propranolol	260.0 $[M+H]^+$	183	60-30-10	116	60-35-8	8.4	1
Propranolol-d7 (S)	267.3 $[M+H]^+$	122.9	86-31-6	-	-	8.3	-
Sotalol	273.0 $[M+H]^+$	213	60-25-6	255	60-25-6	3.9	1
Sotalol-d6 (S)	278.8 $[M+H]^+$	133.9	66-35-4	-	-	3.9	-
Sulfamethazine-d4 (S)	283.1 $[M+H]^+$	186.1	76-25-16	-	-	6.9	-
Sulfadiazine	251.0 $[M+H]^+$	156	71-25-12	92	71-43-0	5.9	1.6
Sulfadiazine-d4 (S)	255.0 $[M+H]^+$	160	71-25-12	-	-	5.7	-

Table S4 – (continues)

Compounds	**Precursor ion (m/z)**	**SRM 1**	**DP-CE-CXP**	**SRM 2**	**DP-CE-CXP**	**Rt (min)**	**SR /SR**
Sulfamethazine	279.0 $[M+H]^+$	124	71-33-10	186	71-25-0	7	2.1
Sulfamethazine-d4 (S)	283.1 $[M+H]^+$	186.1	76-25-16	-	-	6.9	-
Sulfamethoxazole	254.0 $[M+H]^+$	156	71-25-2	92	71-41-6	8.9	1.1
Sulfamethoxazole-d4 (S)	258.0 $[M+H]^+$	96	71-41-6	-	-	8.8	-
Trimethoprim	291.0 $[M+H]^+$	230	76-33-0	261	76-31-20	5.5	1.8

Table S5. Amounts of the pharmaceutically active compounds (PhACs) analyzed in this study consumed in Spain in 2016, in terms of DDD (Daily doses defined, mg active compound/d) and DHD (Daily doses defined per 1000 inhabitant per day), with the excretion factor E.

PhACs (by therapeutic group)	DDD (mg DHD^{-1} d^{-1})	DHD (DDD 1000 inh^{-1} d^{-1})	E
Antibiotics			
Ofloxacin	400	0.004	0.8[c]
Clarithromycin	750	0.782	0.25[a,b]
Sulfamethoxazole	2000	0.294	0.3[c]
Trimethoprim	400	0.059	0.8[a]
Sulfadiazine	600	0.006	n. d.
Sulfamethazine	4000	0.001	n. d.
β-blockers			
Atenolol	75	7.64	0.90[c]
Propranolol	160	0.64	0.10[c]
Sotalol	160	0.160	1.00[c]
Metoprolol	150	0.390	0.07[c]
Antihypertensive/diuretics			
Furosemide	40	10.66	0.45[c]
Hydrochlorothiazide	25	7.81	0.95[c]
Lipid regulators			
Gemfibrozil	1200	1.71	0.20[c]
Bezafibrate	600	0.15	0.40[c]
Fenofibrate	200	4.21	n.d.
Psychiatric medications			
Lorazepam	2.5	21.99	0.73[c]
Carbamazepine	1000	0.98	0.03[a]
Paroxetine	20	11	n.d.
Diazepam	10	7.81	0.01[b]

[a] Verlicchi et al. (2010); [b] Al Aukidy et al. (2012); [c] Jelic et al. (2014); n.d.: no data available.
* Value calculated with 100 % (1.00) excretion factor.

Predicted daily mass load (PL, mg day^{-1} 1000 inh^{-1}) calculation of the target PhACs in influent of the A^{2}O system.

The predicted daily mas load (PL) was calculated by Eq. (1). DHD is the information of daily doses consumed in Spain per 1000 inhabitant (DDD 1000 inh^{-1} d^{-1}) reported by the Spanish Agency of Medicines and Healthcare Products and the Spanish National Plan of Resistance to Antibiotics(PRAN), the DDD (Daily doses defined, mg active compound day^{-1}) reported by WHO Collaborating Centre for Drug Statistics Methodology (WHO) and the excretion ratio for every compound reported in the literature.

$$PL\left(\frac{mg}{1000\ inh.day}\right) = DDD \times DHD \times E \quad \textbf{(1)}$$

Table S6. Spearman rank-correlation coefficients (ρ) between the enviromental/operational variables in the A^2O system
bezafibrate, clarithromycin, gemfibrozil, hydrochlorothiazide, and sotalol. Abiotic variables: biological oxygen demand at f
(COD), food-to-microorganisms ratio (F/M), mixed-liquor suspended solids (MLSS), NH_4^+, nitrogen removal rate (NRR
temperature (OT), phosphorous removal rate (PRR), total N (TN), solids retention time (SRT), and sludge volumetric index (
(*: $p<0.05$; **: $p < 0.10$).

PhACs	BOD	COD	F/M	MLSS	NH_4^+	NRR	ORR	OT	PI
Bezafibrate	0.20	0.18	**-0.78***	**0.62****	**0.78***	**0.88***	**0.73***	-0.58	**0.**
Clarithromycin	0.38	0.35	**-0.65****	**0.61****	**0.78***	**0.73***	0.51	-0.50	0
Gemfibrozil	0.25	0.22	**-0.76***	**0.73***	**0.67****	**0.68****	0.53	-0.44	**0.**
Hydrochlorothiazide	-0.41	-0.40	**0.63****	**-0.83***	**-0.65****	**-0.81***	-0.36	0.47	**-0.6**
Sotalol	-0.52	-0.45	0.51	**-0.72***	**-0.65****	-0.49	-0.54	0.27	**-0.**

Table S7. Number of total reads, average phylotypes (PHYs) richness (S), Good's coverage, and average plus standard deviation of the diversity indices Chao-1, Shannon-Wiener (*H'*) and Simpson's evenness, calculated from the Illumina sequencing data of Bacteria, Archaea and Fungi communities in the A^2O system in experimental phases I and II.

Target	Phase	No. of total reads	Average Richness (S)	Good's coverage (%)	Chao-1 richness estimation	Shannon-Wiener (*H'*)	Simpson
Bacteria	**I**	69070	587.5 ± 51.7	99	733.5 ± 71.9	6.4 ± 0.2	1.0 ± 0.00
	II	51687	580.7 ± 84.1	98	769.2 ± 121.2	6.2 ± 0.2	0.94 ± 0.01
Archaea	**I**	29976	45.0 ± 16.6	99	47.3 ± 16.9	3.6 ± 0.3	0.9 ± 0.10
	II	15216	35.5 ± 11.8	99	39.9 ± 12.6	3.7 ± 0.3	0.89 ± 0.03
Fungi	**I**	4922	49.8 ± 19.6	96	53.1 ± 18.6	4.5 ± 0.5	0.9 ± 0.00

Table S8. Phylotypes (PHYs) which mostly contributed to the global similarity of the bacterial communities between samples of phases I (A) and phase II, according to SIMPER analysis. (B). (C) Phylotypes (PHYs) which mostly contribute to the dissimilarity of the total bacterial communities between phases I and II, according to SIMPER analysis.

A.

Phase I Average similarity among samples of phase I: 61.48%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY 406	*Cytophagaceae*	14.01	15.67	15.67
PHY 962	*Comamonadaceae*	6.23	8.39	24.06
PHY 630	*Xanthomonadaceae*	4.33	5.32	29.38
PHY 713	*Caldilineaceae*	3.41	4.37	33.75
PHY 118	*Caldilineaceae*	3.11	4.29	38.04
PHY 662	*Xanthomonadaceae*	2.52	3.60	41.64
PHY 715	*Chromatiaceae*	3.24	2.77	44.41

B.

Phase II Average similarity among samples of phase II: 71.09%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY 962	*Comamonadaceae*	16.54	18.97	18.97
PHY 118	*Caldilineaceae*	6.65	8.36	27.33
PHY 406	*Cytophagaceae*	9.18	8.34	35.67
PHY 778	*Rhodocyclaceae*	3.70	4.31	39.98
PHY 713	*Caldilineaceae*	4.20	4.27	44.25
PHY 1004	*Comamonadaceae*	2.47	2.41	46.66
PHY 312	Unclasiffied *Myxococcales*	2.09	1.68	48.35

C.

Average dissimilarity among phases I and II: 47.93%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance, phase I**	**% Average relative abundance, phase II**	**% Cumulative contribution**
PHY 962	*Comamonadaceae*	6.23	16.58	10.80
PHY 406	*Cytophagaceae*	14.01	9.18	7.53
PHY 118	*Caldilineaceae*	3.11	6.65	3.69
PHY 778	*Rhodocyclaceae*	0.22	3.70	3.63
PHY 715	*Chromatiaceae*	3.24	0.11	3.26
PHY 630	*Xanthomonadaceae*	4.33	1.54	2.91
PHY 972	Unclassified *Betaproteobacteria*	2.67	0.95	2.55

Table S9. Phylotypes (PHYs) which mostly contributed to the global similarity of Archaea communities between samples of phases I (A) and II (B), according to SIMPER analysis. (C) Phylotypes (PHYs) which mostly contribute to the dissimilarity of the Archaea communities between phases I and II, according to SIMPER analysis.

A.

Phase I Average similarity among samples: 61.95%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance**	**% Average similarity among samples**	**% Contribution**
PHY 87	*Methanolinea*	23.08	14.72	23.76
PHY 130	*Methanobrevibacter*	14.19	11.56	18.65
PHY 29	*Methanobacterium*	12.52	11.44	18.46
PHY 25	*Methanosaeta*	9.25	6.24	10.07
PHY 49	*Methanospirillum*	5.06	3.41	5.51
PHY 116	*Methanospirillum*	3.56	2.85	4.59
PHY 15	*Methanolinea*	5.20	2.70	4.36

B.

Phase II Average similarity among samples: 48.70%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance**	**% Average similarity among samples**	**% Contribution**
PHY 29	*Methanobacterium*	14.89	12.47	25.60
PHY 130	*Methanobrevibacter*	13.98	9.21	18.91
PHY 41	*Methanomethylovorans*	6.91	5.93	12.17
PHY 87	*Methanolinea*	8.86	4.07	8.35
PHY 129	*Methanospirillum*	4.22	3.06	6.27
PHY 49	*Methanospirillum*	4.81	2.14	4.38
PHY 116	*Methanospirillum*	3.65	2.11	4.34

C.

Average dissimilarity among phases I and II: 47.31%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance, phase I**	**% Average relative abundance, phase II**	**% Contribution**
PHY 87	*Methanolinea*	23.08	8.86	16.34
PHY 25	*Methanosaeta*	9.25	5.15	7.79
PHY 130	*Methanobrevibacter*	14.19	13.98	6.85
PHY 59	*Methanospirillum*	0.50	5.29	5.85
PHY 15	*Methanolinea*	5.20	0.00	5.50
PHY 73	*Nitrososphaera*	0.01	4.55	4.81
PHY 41	*Methanomethylovorans*	3.87	6.91	4.77
			Cumulative % contribution	**51.91**

Table S10. Phylotypes (PHYs) which mostly contributed to the global similarity of the fungal communities between samples of phase I.

Average similarity among samples of phase I: 39.30%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance**	**% Average similarity among samples**	**% Contribution**
PHY 55	*Eurotiales*	6.77	6.03	15.33
PHY 73	*Hypocreales*	8.69	4.80	12.20
PHY 38	*Eurotiales*	4.99	3.75	9.54
PHY 85	*Trichosporonales*	5.08	3.63	9.23
PHY 62	*Saccharomycetales*	8.09	3.63	9.23
PHY 56	*Saccharomycetales*	4.22	1.71	4.34
PHY 3	*Trichosporonales*	2.72	1.55	3.94
PHY 89	*Mortierellales*	2.77	1.29	3.27
PHY 80	*Blastocladiales*	2.99	1.22	3.11
PHY 77	*Eurotiales*	1.93	1.19	3.02

Table S11. Pearson product-moment correlation coefficients (r) between the vectors in Figure 2, which represent the relati
and Archaea (B) taxa detected by Illumina-sequencing in the biotic samples from the experimental phases I and II and the f
at five days (BOD_5), chemical oxygen demand (COD), food-to-microorganisms ratio (F/M), mixed-liquor suspended so
(NRR), organic removal rate (ORR), operational temperature (OT), phosphorous removal rate (PRR), solids retention time
total N (TN). Correlations ≥ 0.50 or ≤ -0.50 are boldfaced.

A. Bacteria	**BOD_5**	**COD**	**F/M**	**MLSS**	**NH_4^+**	**NRR**	**ORR**	**O**
Alphaproteobacteria								
Hyphomicrobiaceae	**0.93**	**0.98**	**-0.91**	**0.98**	**0.89**	**0.74**	**0.91**	**-1.0**
Rhodobacteraceae	**0.95**	**0.99**	**-0.88**	**0.96**	**0.92**	**0.78**	**0.88**	**-1.0**
Sphingomonadaceae	**0.89**	**0.96**	**-0.95**	**0.99**	**0.84**	**0.66**	**0.95**	**-0.9**
Unclassified *Rhizobiales*	**0.79**	**0.89**	**-0.99**	**1.00**	**0.72**	**0.51**	**0.99**	**-0.9**
Unclassified *Sphingomonadales*	**0.92**	**0.98**	**-0.93**	**0.98**	**0.87**	**0.71**	**0.93**	**-0.9**
Betaproteobacteria								
Comamonadaceae	**0.96**	**1.00**	**-0.86**	**0.95**	**0.93**	**0.80**	**0.86**	**-1.0**
Oxalobacteraceae	0.35	0.16	0.42	-0.24	0.44	**0.66**	-0.43	-0.0
Rhodocyclaceae	**0.86**	**0.94**	**-0.97**	**1.00**	**0.81**	**0.62**	**0.96**	**-0.9**
Unclassified *Thiobacterales*	**0.99**	**1.00**	**-0.80**	**0.90**	**0.97**	**0.87**	**0.79**	**-0.9**
Unclassified *Betaproteobacteria*	**0.52**	0.34	0.25	-0.05	**0.60**	**0.79**	-0.25	-0.2
Gammaproteobacteria								
Chromatiaceae	**-0.93**	**-0.98**	**0.92**	**-0.98**	**-0.89**	**-0.73**	**-0.91**	**0.9**
Xanthomonadaceae	**-0.96**	**-1.00**	**0.87**	**-0.95**	**-0.93**	**-0.80**	**-0.87**	**1.0**
Bacteroidetes/Chlorobi								
Chitinophagaceae	0.26	0.06	**0.51**	-0.33	0.35	**0.59**	**-0.52**	0.0
Cytophagaceae	**-0.94**	**-0.85**	0.41	**-0.58**	**-0.97**	**-1.00**	-0.41	**0.8**
Flavobacteriaceae	**0.93**	**0.98**	**-0.91**	**0.97**	**0.89**	**0.74**	**0.91**	**-1.0**
Saprospiraceae	**-0.94**	**-0.99**	**0.90**	**-0.97**	**-0.90**	**-0.75**	**-0.90**	**1.0**
Unclassified *Chlorobi*	**-0.68**	**-0.81**	**1.00**	**-0.98**	**-0.61**	-0.37	**-1.00**	**0.8**
Unclassified *Sphingobacteriales*	**-0.91**	**-0.98**	**0.93**	**-0.98**	**-0.87**	**-0.70**	**-0.93**	**0.9**
Chloroflexi								
Caldilineaceae	**1.00**	**0.99**	**-0.75**	**0.86**	**0.99**	**0.91**	**0.74**	**-0.9**

Table S11 (continues)

B. Archaea	**BOD_5**	**COD**	**F/M**	**MLSS**	**NH_4^+**	**NRR**	**ORR**	**O**
Euryarchaeota-Methanobacteriales								
Methanobacterium	**1.00**	**0.90**	-0.43	**0.97**	**0.68**	**0.96**	0.23	**-0.8**
Methanobrevibacter	0.34	**0.77**	**-1.00**	**0.64**	**0.95**	**0.66**	**0.98**	**-0.8**
Methanosphaera	**0.56**	**0.91**	**-0.97**	**0.81**	**1.00**	**0.83**	**0.90**	**-0.9**
Unclassified *Methanobacteriales*	**-0.58**	**-0.92**	**0.97**	**-0.82**	**-1.00**	**-0.84**	**-0.89**	**0.9**
Euryarchaeota-Methanomicrobiales								
Methanocorpusculum	**-0.97**	**-0.95**	**0.56**	**-0.99**	**-0.78**	**-0.99**	-0.37	**0.8**
Methanoculleus	**0.91**	**0.57**	0.06	**0.72**	**0.23**	**0.69**	-0.27	-0.4
Methanofollis	**0.62**	0.13	**0.51**	0.32	-0.24	0.28	**-0.68**	0.0
Methanolinea	-0.34	**-0.77**	**1.00**	**-0.64**	**-0.95**	**-0.66**	**-0.98**	**0.8**
Methanoregula	**0.72**	**0.98**	**-0.90**	**0.91**	**0.99**	**0.93**	**0.79**	**-1.0**
Methanospirillum	**-0.92**	**-0.59**	-0.04	**-0.73**	-0.25	**-0.71**	0.25	0.4
Unclassified *Methanoregulaceae*	0.46	-0.07	**0.67**	0.13	-0.42	0.09	**-0.81**	0.2
Unclassified *Methanospirillaceae*	**-0.69**	**-0.97**	**0.92**	**-0.90**	**-0.99**	**-0.91**	**-0.82**	**1.0**
Unclassified *Methanomicrobiales*	0.15	**0.64**	**-0.98**	0.47	**0.87**	**0.51**	**1.00**	**-0.7**
Euryarchaeota-Methanosarcinales								
Methanomethylovorans	**0.73**	**0.98**	**-0.90**	**0.92**	**0.99**	**0.94**	**0.78**	**-1.0**
Methanosaeta (*Methanothrix*)	**-0.84**	**-1.00**	**0.80**	**-0.98**	**-0.94**	**-0.98**	**-0.66**	**0.9**
Methanosarcina	**0.99**	**0.78**	-0.22	**0.88**	0.49	**0.87**	0.01	**-0.6**
Thaumarchaeota								
Nitrososphaera	**1.00**	**0.89**	-0.42	**0.96**	**0.67**	**0.95**	0.22	**-0.7**
Unclassified *Nitrososphaeraceae*	**-0.72**	**-0.98**	**0.90**	**-0.91**	**-0.99**	**-0.93**	**-0.79**	**1.0**
Diaphorarchaea group								
Unclassified *Thermoplasmata*	**0.72**	**0.98**	**-0.90**	**0.91**	**0.99**	**0.93**	**0.79**	**-1.0**

Table S12. Pearson product-moment correlation coefficients (r) between the vectors in Figure 2, which represent the relativ Archaea (B) and Fungi (C) taxa detected by Illumina-sequencing in the biotic samples from the experimental phases I and of AIAPS (acetaminophen, ibuprofen, ketoprofen, naproxen), antibiotics (clarithromycin, trimethoprim), diurectics (fur (atenolol) and lipid-regulators (bezafibrate, gemfibrozil). Correlations ≥ 0.50 or ≤ -0.50 are boldfaced

A . Bacteria	Acetaminophen	Ibuprofen	Ketoprofen	Naproxen	Clarithromycin	Trimethoprim	Atenolol	Furosem
Alphaproteobacteria								
Hyphomicrobiaceae	**-1.00**	-0.24	0.39	-0.12	0.38	0.29	0.00	0
Rhodobacteraceae	**-0.71**	**0.58**	-0.45	**0.68**	**0.95**	**-0.54**	**0.76**	**0**
Sphingomonadaceae	**-0.81**	0.45	-0.31	**0.56**	**0.89**	-0.41	**0.65**	**0**
Unclassified *Rhizobiales*	**-1.00**	-0.09	0.24	0.04	**0.51**	0.14	0.15	0
Unclassified *Sphingomonadales*	**-0.99**	-0.05	0.20	0.08	**0.55**	0.10	0.19	**0**
Betaproteobacteria								
Comamonadaceae	-0.45	**0.81**	**-0.71**	**0.88**	**1.00**	**-0.78**	**0.93**	**1**
Oxalobacteraceae	**-0.65**	**0.65**	**-0.52**	**0.74**	**0.97**	**-0.61**	**0.81**	**0**
Rhodocyclaceae	-0.32	**0.88**	**-0.80**	**0.94**	**0.99**	**-0.86**	**0.97**	**1**
Unclassified *Thiobacterales*	0.24	**1.00**	**-1.00**	**0.98**	**0.76**	**-1.00**	**0.95**	**0**
Unclassified *Betaproteobacteria*	0.32	**0.99**	**-1.00**	**0.96**	**0.71**	**-0.99**	**0.92**	**0**
Gammaproteobacteria								
Chromatiaceae	**0.51**	**-0.76**	**0.65**	**-0.84**	**-1.00**	**0.73**	**-0.89**	**-0**
Xanthomonadaceae	**0.63**	**-0.66**	**0.53**	**-0.75**	**-0.98**	**0.62**	**-0.82**	**-0**
Deltaproteobacteria								
Unclassified *Myxococcales*	**1.00**	0.07	-0.23	-0.05	**-0.53**	-0.12	-0.17	-0
Bacteroidetes/Chlorobi								
Chitinophagaceae	**0.63**	**0.87**	**-0.93**	**0.80**	0.41	**-0.89**	**0.72**	0
Cytophagaceae	**0.61**	**-0.68**	**0.55**	**-0.76**	**-0.98**	**0.64**	**-0.83**	**-0**
Flavobacteriaceae	**0.71**	**0.81**	**-0.89**	**0.73**	0.32	**-0.84**	**0.65**	0
Saprospiraceae	**0.80**	**0.72**	**-0.82**	**0.63**	0.18	**-0.76**	**0.54**	0
Unclassified *Chlorobi*	**0.96**	0.42	**-0.55**	0.30	-0.20	-0.46	0.19	-0
Unclassified *Sphingobacteriales*	**0.95**	-0.16	0.00	-0.28	**-0.71**	0.11	-0.39	**-0**
Chloroflexi								
Caldilineaceae	**-0.54**	**0.74**	**-0.63**	**0.82**	**0.99**	**-0.71**	**0.88**	**0**

Table S12 (continues)

B. Archaea	Acetaminophen	Ibuprofen	Ketoprofen	Naproxen	Clarithromycin	Trimethoprim	Atenolol	Furosem
Euryarchaeota-Methanobacteriales								
Methanobacterium	-0.27	0.49	-0.27	**0.57**	**0.56**	-0.08	**0.65**	**0**
Methanobrevibacter	**0.71**	**1.00**	**0.71**	**1.00**	**1.00**	**-0.91**	**0.98**	0
Methanosphaera	**0.92**	**0.93**	**0.92**	**0.89**	**0.89**	**-1.00**	**0.84**	-0
Unclassified *Methanobacteriales*	0.14	**-0.60**	0.14	**-0.67**	**-0.67**	0.22	**-0.75**	**-0**
Euryarchaeota-Methanomicrobiales								
Methanocorpusculum	**0.86**	0.24	**0.86**	0.15	0.15	**-0.62**	0.05	**-0**
Methanoculleus	**-1.00**	**-0.71**	**-1.00**	**-0.64**	**-0.64**	**0.94**	**-0.55**	**0**
Methanofollis	**-1.00**	**-0.71**	**-1.00**	**-0.64**	**-0.64**	**0.94**	**-0.55**	**0**
Methanolinea	-0.29	**-0.88**	-0.29	**-0.92**	**-0.92**	**0.61**	**-0.96**	**-0**
Methanoregula	**0.89**	**0.95**	**0.89**	**0.91**	**0.92**	**-0.99**	**0.87**	-0
Methanospirillum	**0.99**	**0.62**	**0.99**	**0.55**	**0.55**	**-0.89**	0.46	**-0**
Unclassified *Methanoregulaceae*	**-0.90**	**-0.94**	**-0.90**	**-0.90**	**-0.91**	**1.00**	**-0.86**	0
Unclassified *Methanospirillaceae*	**-0.89**	**-0.95**	**-0.89**	**-0.91**	**-0.92**	**0.99**	**-0.87**	0
Unclassified *Methanomicrobiales*	**0.71**	**1.00**	**0.71**	**1.00**	**1.00**	**-0.91**	**0.98**	0
Euryarchaeota-Methanosarcinales								
Methanomethylovorans	-0.11	**0.62**	-0.11	**0.70**	**0.69**	-0.25	**0.77**	**0**
Methanosaeta	**0.83**	**0.98**	**0.83**	**0.96**	**0.96**	**-0.97**	**0.92**	0
Methanosarcina	**-0.64**	0.09	**-0.64**	0.19	0.18	0.33	0.28	**0**
Thaumarchaeota								
Nitrososphaera	**-0.51**	0.24	**-0.51**	0.33	0.33	0.18	0.43	**1**
Unclassified *Nitrososphaeraceae*	-0.45	**-0.95**	-0.45	**-0.97**	**-0.97**	**0.73**	**-0.99**	**-0**
Diaphorarchaea group								
Unclassified *Thermoplasmata*	-0.32	**-0.89**	-0.32	**-0.93**	**-0.93**	**0.63**	**-0.96**	**-0**

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❖ CHAPTER 3

Insights into the removal of pharmaceutically active compounds from sewage sludge by two-stage mesophilic anaerobic digestion

Este capítulo ha sido enviado para su revisión y posible publicación a "Science of the Total Environment".

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Abstract

The removal efficiencies (REs) of twenty-seven pharmaceutically active compounds (PhACs) (eight analgesic/anti-inflammatories, six antibiotics, four β-blockers, two antihypertensives/diuretics, three lipid regulators and four psychiatric drugs) were evaluated in a pilot-scale two-stage mesophilic anaerobic digestion (MAD) system treating thickened sewage sludge from a pilot-scale A_2O^{TM} wastewater treatment plant (WWTP) which was fed with wastewater from the pre-treatment of the full-scale WWTP Murcia Este (Murcia, Spain). The MAD system was long-term operated using two different sets of sludge retention times (SRTs) for the acidogenic (AcD) and methanogenic (MD) digesters (phase I, 2 and 12 days; and phase II, 5 and 24 days, in AcD and MD, respectively). Quantitative PCR (qPCR) and Illumina MiSeq sequencing were used to estimate the absolute abundance of Bacteria, Archaea and Fungi and investigate the structure, diversity and population dynamics of their communities in the AcD and MD effluents. The extension of the SRT from 12 (phase I) to 24 days (phase II) in the MD was significantly linked with an improved removal of carbamazepine, clarithromycin, codeine, gemfibrozil, ibuprofen, lorazepam and propranolol. The absolute abundances of total Bacteria and Archaea were higher in the MD regardless of the phase, while the diversity of bacterial and archaeal communities was lower in phase II, in both digesters. Non-metric multidimensional scaling (MDS) plots showed strong negative correlations among phyla *Proteobacteria* and *Firmicutes* and between genera *Methanosaeta* and *Methanosarcina* throughout the full experimental period. Strong positive correlations were revealed between the relative

abundances of *Methanospirillum* and *Methanoculleus* and the methanogenesis performance parameters (volatile solids removal, CH_4 recovery rate and $\%CH_4$ in the biogas), which were also related to longer SRT. The REs of several PhACs (naproxen, ketoprofen, ofloxacin, fenofibrate and atenolol) correlated positively ($r>0.75$) with the relative abundances of specific bacterial and archaeal groups, suggesting their participation in biodegradation/biotransformation pathways.

1. Introduction

Urban wastewater is the main sink of pharmaceutically active compounds (PhACs) after their consumption by human populations. During urban wastewater treatment, some of these substances are efficiently removed from raw wastewater, but most of them remain in the treated water, sewage sludge, or both (Jelić et al., 2012; Martín et al., 2012; Radjenović et al., 2009; Tran et al., 2018; Yan et al., 2014a).

The development of analytical techniques for the extraction, clean-up and quantification of PhACs from environmental samples has contributed to increase the knowledge about the occurrence and fate of these substances not only in the dissolved phase of raw and treated wastewater, but also in sewage and treated sludge (Gago-Ferrero et al., 2015; Martín-Pozo et al., 2019). For a given PhAC, the concentrations detected in sewage sludge usually range from tens to several hundred $\mu g\ kg^{-1}$ dry weight (dw), and are often related to those measured in the raw wastewater; however, other factors influence the amounts occurring in this complex matrix, such as the physical-chemical properties of each particular compound, their resistance to biodegradation, or the sludge characteristics and operating parameters of each wastewater treatment plant (WWTP) (Carballa et al., 2008; Radjenović et al., 2009; Samaras et al., 2014; Stasinakis, 2012).

Anaerobic digestion (AD) is one of the most effective methods for sewage sludge stabilization, as well as an efficient and environmentally friendly technology for bio-natural gas production (Tezel et al., 2011; Zhang et al., 2016). In particular, the two-stage AD configuration, which relies on the separation of the hydrolysis/acidification and acetogenesis/methanogenesis processes in two different bioreactors, in order to provide optimal conditions for the main microbial groups involved in each of these processes (acid-producing bacteria and methanogenic archaea, respectively), offers some advantages for the stabilization of sewage sludge compared to the single-stage AD configuration, such as improved biogas production, pH self-adjusting capacity, reduction of pathogens and chemical oxygen demand (COD) concentrations, ability to work at shorter sludge retention

times (SRT), and improved microbiota stabilization, among others (Maspolim et al., 2015b; G. Wang et al., 2018).

In Europe, almost half of the sludge generated in WWTPs and stabilized by AD processes is used in agriculture, either directly or after composting (Kelessidis and Stasinakis, 2012). The entry of PhACs into the environment through the application of stabilized sludge as soil fertilizer/conditioner implies a clear risk of contamination not only for environmental compartments (soil and water), but also for human health, particularly due to the spread and maintenance of antibiotic-resistant genes (ARG) and antibiotic resistant bacteria (aus der Beek et al., 2016; Y. Wu et al., 2016). However, only few countries of the EU set in their national legislation limit values of concentrations for some target compounds in stabilized sludge as a requisite for land spreading, and to date no PhACs have been included among these (Christodoulou and Stamatelatou, 2016; Martín-Pozo et al., 2019).

The removal of PhACs such as non-steroidal anti-inflammatory drugs, antibiotics or the antiepileptic drug carbamazepine from sewage sludge in laboratory or full-scale AD systems have been previously reported (Carballa et al., 2007b; Martín et al., 2015; Narumiya et al., 2013; Samaras et al., 2014, 2013; Stasinakis, 2012; Zhou et al., 2018, 2017, 2015). At the same time, several investigations based on molecular methods have been carried out to provide an insight of the community structure and populations' diversity of the microbiota involved in the processes (hydrolysis, acidogenesis, acetogenesis and methanogenesis) that are accomplished in sewage sludge AD working under several configurations and operation conditions (Hao et al., 2016; Ma et al., 2019; Maspolim et al., 2015b). Nevertheless, no clear conclusions can be drawn regarding the ranges of PhACs removal efficiencies and the way these are influenced by operation parameters, since the results widely vary among the different studies, and reports providing links between microbial diversity shifts and removal efficiencies are limited, particularly for the two-stage AD systems.

The aim of the present work was twofold: i) to study the occurrence and fate of twenty-seven PhACs belonging to six therapeutic groups (antibiotics, anti-inflammatory/analgesic compounds, β-blockers, antihypertensives/diuretics, lipid regulators and psychiatric drugs) in sludge sewage samples undergoing two-stage mesophilic AD (MAD) treatment, in order to ascertain its effectiveness to remove these emerging contaminants, and ii) to characterize the microbial populations present in the sludge samples under the acidogenic and methanogenic conditions, to gain insight into their

community structure and diversity, as well as to show by statistical tools the existing relationships between the shifts of the microbial populations' diversity, the two-stage MAD performance, and the removal efficiency of PhACs.

2. Materials and methods

2.1. Description of the pilot-scale two-stage MAD plant and operational conditions

The two-stage MAD plant consisted of two stainless-steel cylindrical digesters (acidogenic and methanogenic, connected in series) with a volume of 640 L and 2340 L, respectively, and operated under mesophilic conditions at different SRTs (Figure S1). The external surface of the digesters was wrapped with a thermal jacket to maintain constant mesophilic conditions (35 ± 0.5 ºC) and a recirculation pump was operated in each digester to maintain the homogeneity of the sludge. A drum-type gas flow meter (TG3 plastic, Ritter Company) and a pH meter (GRLP 22, Crison) were installed to monitor these variables in the digesters.

The two-stage MAD plant was designed for processing the primary sludge (PS) and waste activated sludge (WAS) generated in a pilot-scale anaerobic-anoxic-aerobic (A^2O) system (Figure S2) installed at the facilities of WWTP *Murcia Este* (EMUASA, Murcia, Spain), which was fed with wastewater from the pre-treatment of the full-scale WWTP. The operation parameters of the A^2O bioreactor and the characteristics of the activated sludge have been previously described in full detail by Gallardo-Altamirano et al. (2018). Both the A^2O and the two-stage MAD were equipped with a supervisory control and data acquisition (SCADA) system to control the operational parameters.

2.2. Inoculation and experimental set-up of the two-stage MAD plant

Digested sludge (200 L) from WWTP *Murcia Este* (EMUASA, Murcia, Spain) was used as biomass inoculum for acidogenic and methanogenic digesters of the two-stage MAD plant. The primary and secondary sludge generated in the water treatment line of the A^2O system were mixed and thickened in a gravitational thickener (216 L volume, 1.45 m high and 0.5 m diameter, Figure S2) and then used as feeding for the two-stage MAD plant. Since June 1st 2016, the A^2O system was working under steady-state conditions, producing a regularly thickened sludge (ThS) at a flow of 41 ± 9 L d^{-1}. Thereby, the acidogenic and methanogenic digesters (AcD and MD, respectively) started up operating at a constant organic loading rate (OLR) and at the SRTs selected for phase I (2 days for the AcD and

12 days for the MD). Both digesters were operated until steady-state conditions were reached, before experimental phase I started, which lasted 104 days (July 27th - November 13th 2016). Then, operating conditions for the experimental phase II were fixed at SRTs of 5 and 24 days for AcD and MD, respectively. Phase II lasted 105 days (December 14th 2016- March 31th 2017).

2.3. Physicochemical parameters and monitoring of the two-stage MAD plant performance

Temperature, pH, and biogas production were measured daily in each digester during both experimental phases. Biogas composition was characterized with a gas analyzer (Siemens Ultramat 23) and was verified once per month with a portable gas analyzer (Geotechnical Instrument Ltd, GA5000).

Sludge samples were taken three times a week from the sampling points 1, 2, and 3 (Figure S2) in order to evaluate the two-stage MAD plant performance. The parameters analysed were: total solids (TS), volatile solids (VS), volatile fatty acids (VFA) and alkalinity (ALK). TS and VS were measured according to Standard Methods (Baird and Bridgewater, 2017); volatile solids removal (VSR) in each digester (AcD or MD) was calculated as follows: %VSR = [(VS influent - VS effluent)/VS influent] × 100 (Bhattacharya et al., 1996); and volatile fatty acids (VFA) and alkalinity (ALK) concentrations were measured by titration, according to the method proposed by DiLallo and Albertson (1961). Tables 1A and 1B summarise the operational conditions of the digesters and physicochemical parameters analysed in each experimental phase, respectively.

2.4. Sample collection for PhACs analysis

A total of 27 sludge samples were collected for PhACs analysis from sample points 1, 2 and 3 (Figure S2): 12 samples in phase I and 15 samples in phase II. From each point, integrated samples (6 L) were taken throughout the day and thoroughly mixed before retrieving a 500 ml homogeneous sample for the PhACs analyses. Samples were collected both midweek and at the end of the weekend, stored in amber PET bottles, and frozen at -20ºC until their analysis. All samples were collected under dry weather conditions to eliminate sample dilution effect.

2.5. Analytical methods for PhACs

2.5.1. Chemicals, reagents and solutions

High-purity (mostly 90%) analytical reference standards (acetaminophen, atenolol, bezafibrate, carbamazepine, clarithromycin, codeine, diazepam, diclofenac, fenofibrate, furosemide, gemfibrozil, hydrochlorothiazide, ibuprofen, indomethacin, ketoprofen, lorazepam, metoprolol, naproxen, ofloxacin, paroxetine, propanolol, propyphenazone, sotalol, sulfadiazine, sulfamethazine, sulfamethoxazole, trimethoprim) were acquired from Sigma Aldrich (St. Luis, MO, U.S). Isotope-labelled compounds (acetaminophen-d4, bezafibrate-d4, carbamazepine-d10, codeine-d3, diazepam-d5, diclofenac-d4, furosemide-d5, fenofibrate-d6, gemfibrozil-d6, ketoprofen-d3, indomethacin-d4, lorazepam-d4, ofloxacine-d3, metoprolol-d7, naproxen-d3, paroxetine-d4, propranolol-d7, sotalol-d6, sulfamethoxazole-d4, sulfadiazine-d4, sulfamethazine-d4) were purchased from Cerilliant, Alsachim (Illkirch-Graffenstaden, France) or Santa Cruz Biotechnology (Dallas, TX, USA). CAS numbers, molecular formulas, molecular weight, and other relevant properties of all target compounds are reported in Gallardo-Altamirano et al. (2019).

LC-MS grade acetonitrile (ACN) (≥ 99.9%), methanol (MeOH) (≥ 99.9%), ethyl acetate (EtAc) (≥ 99.9%), dimethyl sulfoxide (DMSO) (≥ 99.9%), and HPLC water were purchased from Merck (Darmstadt, Germany). Formic acid (≥ 96%, ACS reagent) and ammonium acetate were supplied by Sigma-Aldrich.

Individual stock standard solutions (concentration of 1000 µg mL^{-1}) were prepared in either 100% methanol or 100% DMSO, depending on the solubility of each compound. Working solution mixtures containing all the aforementioned analytes and the isotopically labelled compounds (1 µg mL^{-1}), for analysis and calibration purposes, were prepared by diluting adequate volumes of the individual stock solutions with MeOH. All the solutions were stored at -20 °C.

2.5.2. Sample preparation

The extraction and analysis of sludge samples were based on methods previously published by Jelić et al. (2009) and Gago-Ferrero et al. (2015) respectively, with some modifications. Briefly, about 500 ml of sewage sludge were lyophilized for about 10 days until constant weight. Then, 0.5 g freeze-dried and sieved sludge was placed in a 50-mL falcon tube and spiked with 50 ng of the corresponding surrogate mixture, and kept in contact overnight into a fume hood. Five mL of extraction solvent (MeOH–HPLC water 1:2 (v/v)) were added and the tube was vortexed for 1 min. The sample was then extracted

by ultrasonic bath for 10 min. After the first extraction, the tube was centrifuged for 10 min (4000 rpm, 4° C) and the supernatant was collected in a 16-mL glass test tube. This procedure was repeated two more times but the last time, 5 mL of 0.1 % formic acid in MeOH/water 1:1 (v/v) were used as extraction solvent. In total about 15 mL of supernatant were collected. Then, the extract was evaporated to less than 10 mL under a gentle steam of nitrogen at 24 ºC using a TurboVap® LV (Biotage AB, Uppsala, Sweden) to reduce the amount of methanol for the following SPE step. The extract was diluted in 100 mL of HPLC water to ensure a solvent concentration less than 1%. The whole volume of the aqueous solutions was filtered through a 0.45-µm pore size nylon membrane, 47 mm diameter, and transferred to the SPE procedure using Oasis® HLB cartridges (60 mg, 3 mL, Waters Corporation - Milford, MA, USA). For the SPE, the cartridges were pre-conditioned with 5 mL of EtAc and 5 mL of MeOH, and equilibrated with 5 mL of ultrapure water, and afterwards the sample was loaded onto the cartridges at a flow rate of 1 mL min^{-1}. A washing step was conducted with 2 × 3 mL of ultrapure water and, before elution, the cartridges were dried under vacuum aspiration for 30 min. Analytes were eluted with 3 × 3 mL of EtAc:MeOH (1:1, v/v). The extract was evaporated to dryness under a gentle stream of nitrogen and reconstituted with 1 mL of H_2O/MeOH (90:10) and then filtered to 2-mL vials using 0.22 µm polytetrafluoroethylene (PTFE) syringe filters for UPLC-MS/MS analysis.

2.5.3. Liquid-Chromatography (LC) separation

LC separation was performed using a SCIEX ExionLC™ AD system (Sciex, Redwood City, CA, U.S.). Chromatographic separation was achieved on a Hibar® HR Purospher® STAR RP-C18 column (100 mm × 2.1 mm i.d., 2 µm particle size, Merck, Darmstadt, Germany), maintained at 40 °C in the column oven. The mobile phase for the positive electrospray ionization consisted of 5 mM ammonium acetate and 0.1% of formic acid in water (A) and ACN (B), while for the negative electrospray ionization it consisted of 10 mM ammonium acetate in water (A) and MeOH:ACN (1:1, v/v) (B). The total run time for each injection was 24 min for positive and 18 min for negative acquisition with a flow rate of 0.4 mL/min. The injection volume was 5 µL, and the auto-sampler temperature was maintained at 8 °C. The elution gradient for ESI(+) mode started with 5 % of B for 30 sec, then increasing to 98 % in 16 min, held at 98 % for 3 min, and finally returning to the initial conditions in the next 20 sec. The necessary time for the re-equilibration of the analytical column was 5 min. The elution gradient for ESI(−) started with 5 % B. the

organic phase was then increased to 95 % in 12 min and kept constant for 1 min, before a return to the initial conditions over the next 30 sec.

2.5.4. Mass Spectrometry (MS) and MS/MS conditions

The SCIEX X500R QTOF system (Sciex, Redwood City, CA, U.S.) with Turbo V™ source and Electrospray Ionization (ESI) and operating in negative/positive polarity was used for the detection of the compounds of interest. Any drift in the mass accuracy of the SCIEX Q-TOF was automatically corrected and maintained throughout batch acquisition by infusion of reserpine reference standard (C33H40N2O9, m/z 609.28066) for positive ionization, and a cluster of trifluoroacetic acid (5(TFA-Na)+ TFA-, m/z 792.85963) for negative mode. Calibration was running every 5 samples during the batch acquisition making use of the Calibrant Delivery System (CDS).

High resolution data were acquired using a multiple reaction monitoring (MRM^{HR}) workflow consisting of a TOF-MS survey (100-850 Da for 80 ms of Accumulation Time (AT); Declustering Potential (DP) and Collision Energy (CE) were set to 80 V and 10 V and -80 V and -10 V, for positive and negative, respectively. The MRM^{HR} scanning mode was used for accurate quantification of product ion transitions. The Guided MRM^{HR} tool from SCIEX was used for the optimization of high-resolution transitions (Table S1 and S2). The optimized ionization mode, fragmentation voltages, and collision energies for each compound have been supplied as Supplementary material (Tables S1, S2; Figure S3, S4, S5 and S6). The source conditions for the system were optimized as follow. Ion Spray Voltage was set to 5500 V (-4500 V for negative); source temperature and nitrogen gas flows (Atomizing gas, GS1 and Auxiliary gas, GS2) were set to 550° C, 50, and 70 psi, respectively. Curtain gas was set to 35 psi.

Qualitative and quantitative analysis were performed using SCIEX OS™ Software version 1.4 (Sciex, Redwood City, CA, U.S.). Two high resolution product ions were used for each compound, the most abundant for the quantification and the second most abundant for the confirmation.

2.6. DNA extraction, qPCR, massive parallel sequencing and data analysis

Environmental DNA was extracted from sludge samples (4 ml) following already described protocols (Gallardo-Altamirano et al., 2018). The absolute abundances of total Bacteria, Archaea and Fungi were quantified in each of the samples by real-time quantitative PCR (qPCR), using a QuantStudio-3 Real Time PCR system (Applied

Biosystems).The Domain and Phylum-level universal primer pairs used, quantitative amplification reactions and cycling conditions were already described elsewhere (Gallardo-Altamirano et al., 2018).

High-throughput sequencing of the small-subunit rRNA genes of the mixed microbial populations was achieved using the Illumina MiSeq platform (Illumina, Hayward, CA, USA) at the facilities of RTL Genomics (Lubbock, TX, USA, http://www.researchandtesting.com). The primer pairs 28F-519R (Fan et al., 2012) and 517F/909R (Maspolim et al., 2015b) were used to amplify the 16S rRNA genes of Bacteria and Archaea, respectively, while the 18S rRNA gene of Fungi was amplified using the FungiQuantF/FungiQuantR primer set (Liu et al., 2012).

Raw sequencing data were processed using the QIIME software, v. 1.9.1, following the pipeline protocol described by Caporaso et al. (2012). The sequences were filtered and taxonomic assignments were made as described in full detail by Gallardo-Altamirano et al. (2019). Alpha-diversity indices (Chao-1, Shannon-Wiener *H'*, and Simpson) were calculated using PAST v. 3.25 (Hammer et al., 2001). The 'iNEXT' free online tool (https://chao.shinyapps.io/iNEXTOnline, Hsieh et al., 2016) used to construct sample-size-based rarefaction and extrapolation (R/E) sampling curves with 95% confidence intervals. Heatmaps displaying the relative abundances of Bacteria, Archaea and Fungi in the samples were generated using Microsoft Excel.

The Primer software (PRIMER-E v. 6.1.18, Plymouth, UK) was used to construct an UPGMA dendrogram with 95% confidence intervals, and to estimate the contribution of individual PHYs to the dis(similarity) between groups of samples, as already described by Maza-Márquez et al. (2016), by using the SIMPROF and SIMPER commands, respectively.

2.7. Statistical analyses

The Kruskal-Wallis non-parametric test was selected to find significant differences among the different groups of samples in the analyzed data sets, using a 95% significance level ($p < 0.05$). When required, Spearman's rank correlation coefficients (ρ) among the different variables were calculated. All these procedures were done using IBM SPSS Statistics v. 19 (SPSS Inc., IBM, USA). The multivariate statistical analyses (Non-metric multidimensional scaling, MDS; and analysis of similarity, ANOSIM) were performed using the Primer software PRIMER-E v. 6.1.18, Plymouth, UK). Vectors representing the trends through the MDS ordinations of either biotic (absolute or relative abundances of

bacterial populations) or abiotic variables (operational parameters or indicators of methanogenesis efficiency) were generated by multiple partial correlation algorithms and represented over the plots. All the aforementioned procedures were described in full detail elsewhere (Maza-Márquez et al., 2016b).

3. Results and discussion

3.1. Performance of the pilot-scale two-stage (MAD) plant

Table 1A shows the operational conditions and performance of the AcD and MD of the two-stage MAD plant during the two experimental phases of the study. The characterization of the sludge samples (ThS, AcD effluent and MD effluent) is summarized in Table 1B.

The two digesters were operated under mesophilic conditions (35 ºC) in both phase I and II (Table 1A), with similar influent mass flow (1195 ± 213 and 1261 ± 187 g TS d^{-1} ThS, respectively, Table 1 B). However, the ThS had significantly different characteristics in phases I and II, regarding the % TS and % VS/TS ratio (2.96 ± 0.74 vs. 2.88 ± 0.59 and 72.0 ± 2.3 vs. 74.6 ± 3.7 %, respectively, Table 1B) and the contribution of primary sludge (PS) and waste sludge (WAS) (67 ± 15 vs. 57 ± 16 % and 33 ± 15 vs. 43 ± 16 %, respectively, Table 1A). The observed differences could be related to the fact that the water treatment line (A^2O bioreactor) worked under different operational conditions in each experimental phase, particularly with regard to the mixed liquor suspended solids (MLSS) concentration, food-to-microorganism (F/M) ratio and OLR, as previously reported by Gallardo-Altamirano et al. (2018).

In both experimental phases, the average % TS in the ThS (Table 1B) were lower than those usually measured in mixed ThS in full-scale WWTPs (Appels et al., 2008; Metcalf & Eddy, 2003; Ponsá et al., 2008). Thereby, the global OLR applied to the two-stage MAD plant in both experimental phases (1.5 and 0.9 Kg VS·m^{-3} d^{-1} in phase I and II, respectively, Table 1A) was low compared with a typical high-rate wastewater sludge AD (1.6-4.8 Kg VS m^{-3} d^{-1}, Appels et al., 2008).

The values of the performance parameters VSR, biogas production, biogas composition, biogas recovery rate, biogas production rate, biogas yield, VFA, and ALK recorded in the AcD and MD were statistically different between phases I and II (Table 1A). Lower VSR and biogas production values were detected in phase I in both digesters, possibly in relation with the shorter SRTs selected (2 days and 12 days for the acidogenic

and methanogenic digesters, respectively), while in the MD, the biogas and methane recovery rates and the biogas production rate were greater in phase I than in phase II.

In phase II, the AcD (operating at SRT=5 days) consumed a higher amount of biodegradable substrates, resulting in higher VSR (25.5 %), biogas production (224 L d^{-1}) and biogas production rate (1.10 L L^{-1} sludge d^{-1}), and lower VFA concentration (565 mg CH_3COOH L^{-1}), compared to the values obtained in the same digester in phase I (Table 1A). In both experimental phases, the global performance of the mesophilic two-stage MAD plant was in the range of values reported for other similar processes applied to sewage sludge in the earlier literature (Martín-Pascual et al., 2017; Maspolim et al., 2015b; Ponsá et al., 2008; Smith et al., 2017).

The separation of acidogenic and methanogenic microorganisms in the two different digesters was only effective in phase I, while in phase II both digesters yielded similar values of % CH_4 in the biogas and CH_4 production (Table 1A). In phase II, lower VFA/ALK ratio and higher pH were measured in the AcD (Table 1), compared with data of previous studies in two-stage systems (Ghosh, 1987; Martín-Pascual et al., 2017; Maspolim et al., 2015a; Ponsá et al., 2008). Thereby, our results show that an SRT shorter than 5 days is required for an optimal separation between the acidogenic and methanogenic stages when operating at low OLR. In this sense, Ponsá et al. (2008) optimized the AcD stage of a thermophilic (55 ºC) sludge digestion process, concluding that the critical step was the shortening of the SRT from 5 to 4 days, in order to digest a mixed ThS with high concentration of solids (%TS > 4), while the optimal SRT was 1 day for sludge with lower concentration of solids (%TS < 3). Ghosh (1987) reported that the hydrolysis and acidification of the sewage sludge were more efficient at pH 6 and using a SRT of 2 days in the AcD of a mesophilic two-stage digestor. Martín-Pascual et al. (2017), working with digesters of different volumes (20 and 60 L), obtained maximum methane production in the MD of a two-stage system when the SRT of the AcD was 2.18 days, reaching the maximum value of VFA and complete separation of the stages with the lowest digester volume. Otherwise, (Maspolim et al., 2015a) operated 3 mesophilic AcDs at HRTs of 5, 3 and 2 days, achieving the highest VFA production at the longest SRT, although reaching the same % CH_4 in the biogas (48%) in the three AcDs when working at a constant pH value of 5.5.

Table 1. A. Operational conditions and performance of the acidogenic and methanogenic digesters (AcD and MD, mesophilic anaerobic digestion (MAD) process, in the experimental phases I and II. **B.** Characterization of the thickened effluent and methanogenic digester (MD) effluent in the experimental phases I and II. Values shown are means ± SD. significantly different between the two experimental phases (I and II) in each digestion stage (AcD, MD or global two- Wallis test ($p < 0.05$). TS: Total solids; VS: Volatile solids.

A.

	AcD		MD	
Parameter	**Phase I**	**Phase II**	**Phase I**	**Phase II**
SRT, d	2.0 ± 0.6	5.2 ± 1.1	12.3 ± 1.4	24.8 ± 1.4
T, ºC	35 ± 0.6	35 ± 0.7	35 ± 0.5	35 ± 0.5
pH	6.7 ± 0.1*	7.1 ± 0.2*	7.3 ± 0.2*	8.0 ± 0.2*
OLR, Kg VS m^{-3} d^{-1}	11.1 ± 2.1*	4.6 ± 0.6*	1.6 ± 0.4*	0.8 ± 0.4*
% PS, %TS influent	67 ± 15*	57 ± 16*	-	-
% WAS, % TS influent	33 ± 15*	43 ± 16*	-	-
VSR, %	9.6 ± 3.9*	25.5 ± 9.6*	25.7 ± 9.0*	33.7 ± 14*
Biogas production, L d^{-1}	71 ± 19*	224 ± 81*	188 ± 38	211 ± 63
Biogas composition % CH_4	48.2 ± 7.6*	59.5 ±3.3*	65.6 ± 2.6*	54.9 ± 10*
% CO_2	40.6 ± 6.4*	39.1 ± 3.0*	31.5 ± 1.8	32.5 ± 2.3
CH_4 production, L d^{-1}	34 ± 10*	131 ± 46*	122 ± 24	119 ± 40
Biogas production rate, L $L^{-1}d^{-1}$	0.86 ± 0.23*	1.10 ± 0.40*	0.38 ± 0.09*	0.26 ± 0.10*
Biogas recovery rate, m^{-3} Kg VS $removed^{-1}$	0.788 ± 0.111*	0.819 ± 0.007*	0.909 ± 0.075*	0. 871 ± 0.013*
CH_4 recovery rate, m^3 Kg VS $removed^{-1}$	0.380 ± 0.079*	0.487 ± 0.016*	0. 596 ± 0.047*	0.483 ± 0.043*
Biogas yield, m^3 Kg VS fed^{-1}	0.084 ± 0.021*	0.214 ± 0.056*	0. 241 ± 0.049*	0.297 ± 0.067*
CH_4 yield, m^3 Kg VS fed^{-1}	0.041 ± 0.010*	0.126 ± 0.032*	0.157 ± 0.031*	0.167 ± 0.043*
VFA effluent, mg CH_3COOH L^{-1}	795 ± 243*	565 ± 168*	257 ± 140*	157 ± 50*
ALK effluent, mg $CaCO_3$ L^{-1}	2053 ± 321*	2826 ± 377*	2883 ± 101*	3602 ± 329*
VFA/ALK	0.39 ± 0.11*	0.20 ± 0.06*	0.08 ± 0.04*	0.04 ± 0.01*

B.

Phase	**Phase I**			
Sludge sampling point	**ThS**	**AcD**	**MD**	**ThS**
TS flow, g d^{-1}	1195 ± 213	1111 ± 187	895 ± 185*	1261 ± 187
VS flow, g d^{-1}	869 ± 150	780 ± 131	581 ± 118*	949 ± 131
% TS	2.96 ± 0.74*	2.72 ± 0.57	2.20 ±0.32	2.88 ± 0.59*
% VS/TS	72 ± 2.3*	69.8 ± 1.5	65.3 ± 1.7	74.6 ± 3.7*

3.2. Occurrence of PhACs in sludge samples of the two-stage MAD plant

Table 2 displays the mean concentration, standard deviation and detection frequency of 27 targeted PhACs in the ThS, AcD effluent and MD effluent samples of the two-stage MAD plant during the experimental phases I and II.

The detection frequency value in the ThS samples was 100% for 19 and 17 compounds in phase I and II, respectively (Table 2). Among them, 13 compounds (atenolol, carbamazepine, codeine, diclofenac, fenofibrate, gemfibrozil, ibuprofen, ketoprofen, lorazepam, naproxen, ofloxacin, sotalol and trimethoprim) were detected with the highest frequency value (100% of samples) in both phases. Similar trends were recorded in the AcD effluent samples, with 17 compounds in phase I and 16 compounds in phase II displaying 100% detection frequency. However, a lower number of PhACs with 100% detection frequency occurred in the MD effluent samples (12 in both phases).

According to the Kruskal-Wallis test, the mean concentrations of codeine, diazepam, fenofibrate, furosemide, hydrochlorothiazide, paroxetine and propranolol in the ThS samples were statistically different between phases I and II, probably due to the diverse operational conditions also used in the water treatment line (A^2O bioreactor) (Gallardo-Altamirano et al., 2018), which determined different PhACs concentrations in the sludge treatment line. The statistical analyses also revealed significant differences of the average concentrations of several PhACs measured in the effluents of both digesters between the two experimental phases, particularly in the case of the AcD (Table 2) .

The highest mean concentration values in the ThS were observed for ofloxacin (4922 ng g^{-1}), fenofibrate (2623 ng g^{-1}) and paroxetine (835 ng g^{-1}) in phase I, and ibuprofen (1280 ng g^{-1}), naproxen (507 ng g^{-1}) and ofloxacin (475 ng g^{-1}) in phase II. Several PhACs were detected at mean concentration values $\leq$ 15 ng g^{-1} in both experimental phases (bezafibrate, indomethacin and propyphenazone) or in phase II samples only (diazepam, metoprolol and sulfamethazine). The sulfadiazine concentration was below the limit of detection (BLD) only in phase II samples.

Ofloxacin was the PhACs detected at highest concentrations in the three types of sludge samples, although subjected to a high variability (Table S3). Such variability in sewage sludge was previously reported by Narumiya et al. (2013) and Verlicchi and Zambello (2015), reflecting the use of this fluoroquinolone antibiotic to treat outbreaks of infection diseases; in this sense, Coutu et al. (2013) previously reported a clear seasonality in the pattern of detection of ofloxacin concentrations in wastewater.

Otherwise, the highest PhACs mean values in MD effluent samples were measured for fenofibrate (3456 ng g^{-1}) and ofloxacin (3324 ng g^{-1}) in phase I, and ibuprofen in phases I and II (403 and 1595 ng g^{-1}, respectively), while the values of the remaining PhACs were < 300 ng g^{-1} in both experimental phases (Table 2).

The mean concentrations of the targeted PhACs in the three types of sludge samples fell within the ranges measured earlier by several authors (Martín et al., 2012; Narumiya et al., 2013; Phan et al., 2018; Radjenović et al., 2009; Verlicchi and Zambello, 2015; Yang et al., 2016), except for two compounds which were detected at concentrations above (clarithromycin) or below (fenofibrate) those previously reported in the available literature.

As shown in Table 2, the concentrations of most PhACs gradually decreased throughout the two-stage MAD process, except for carbamazepine, diazepam, diclofenac, gemfibrozil, and ibuprofen (in both phases), fenofibrate, furosemide and propranolol (only in phase I), and paroxetine (only in phase II). Analogous trends were previously reported by several authors for some of the aforementioned PhACs (Narumiya et al., 2013; Phan et al., 2018; Radjenović et al., 2009; Yang et al., 2016), except for diazepam, fenofibrate, furosemide, and paroxetine, of which very little information is actually available.

Table 3 shows the daily measured mass loads (ML, mg day^{-1} 1000 inh^{-1}) of the 27 targeted PhACs in the three sludge flows. The total ML of the ThS was more than twofold in phase I (553 mg day^{-1} 1000 inh^{-1}) compared to phase II (254 mg day^{-1} 1000 inh^{-1}). Antibiotics and lipid-regulators displayed the highest ML percentages in phase I (53 and 28 % respectively). while anti-inflammatory and/or analgesic pharmaceuticals (AIAPs) and diuretics had highest ML percentages in phase II (58 and 15 % respectively). Throughout the full two-stage MAD process, the total ML of the final effluent sludge was reduced to 306 mg day^{-1} 1000 inh^{-1} in phase I and 88 mg day^{-1} 1000 inh^{-1} in phase II (44.7 and 65.3%, respectively). The increased reduction observed in phase II was connected to the highest removal efficiencies (REs) achieved for several of the analysed PhACs compared to phase I, as it will be further discussed in Section 3.3.

ML values for PhACs are well characterised in the wastewater treatment lines of WWTPs (Papageorgiou et al., 2016; Park et al., 2017; Verlicchi et al., 2012; Zorita et al., 2009). However, to the best of the authors' knowledge, the present study is the first reporting ML values for PhACs in a sewage sludge treatment line. The data reported here should be taken into consideration, since treated sewage sludges are commonly applied as organic amendments to agricultural lands in many countries.

Table 2. Concentration (ng g^{-1} dry solid) of PhACs in the thickened sludge (ThS), acidogenic digester (AcD) effluent and
the experimental phases I (n=4) and II (n=5). Values shown are means ± SD; detection frequencies of each compound in t
Data marked with an asterisk (*) were significantly different between the two experimental phases (I and II) in each type
to the Kruskal-Wallis test ($p < 0.05$). BLD. Below detection limit.

Therapeutic groups		**ThS**		**AcD**	
		Phase I	**Phase II**	**Phase I**	**Phase II**
AIAPs	Acetaminophen	111 ± 222 (25)	68 ± 114 (100)	12 ± 25 (25)*	238 ± 203 (100)*
	Codeine	20 ± 14 (100)*	98 ± 93 (100)*	14 ± 17 (50)*	88 ± 90 (100)*
	Diclofenac	134 ± 48 (100)	166 ± 141 (100)	401 ± 475 (100)	212 ± 190 (100)
	Ibuprofen	137 ± 254 (100)	1280 ± 1454 (100)	184 ± 267 (100)*	1125 ± 757 (100)
	Indomethacin	2.3 ± 1 (100)	3.9 ± 3.7 (80)	2.2 ± 1.0 (100)	3.6 ± 3.6 (80)
	Ketoprofen	28 ± 36 (100)	165 ± 161 (100)	24 ± 30 (100)*	105 ± 71 (100)*
	Naproxen	57 ± 60 (100)	507 ± 657 (100)	11 ± 19 (100)	65 ± 60 (100)
	Propyphenazone	12 ± 18 (75)	1.0 ± 1.2 (60)	4.7 ± 4 (75)	0.2 ± 0.5 (60)
Antibiotics	Clarithromycin	34 ± 32 (100)	10 ± 9.2 (80)	9.1 ± 9 (75)	5.6 ± 8.5 (40)
	Ofloxacin	4922 ± 4843 (100)	475 ± 377 (100)	32637 ± 59608 (100)	329 ± 299 (100)
	Sulfadiazine	31 ± 38 (50)	BLD	7.1 ± 9 (50)	BLD
	Sulfamethazine	30 ± 35 (50)	0.6 ± 1.2 (20)	1.1 ± 2 (50)	1.0 ± 2.3 (20)
	Sulfamethoxazole	55 ± 41 (100)	22.2 ± 30 (60)	19 ± 19 (75)*	0.0 ± 0.0 (0)*
	Trimethoprim	19 ± 12 (100)	27.9 ± 7.8 (100)	2.0 ± 2 (75)*	6.6 ± 2.8 (100)*
Beta-blockers	Atenolol	24 ± 3 (100)	49 ± 54 (100)	17 ± 7 (100)	25 ± 21 (100)
	Metoprolol	122 ± 82 (75)	1.8 ± 2.8 (40)	14 ± 9 (100)*	0.3 ± 0.6 (20)*
	Propranolol	86 ± 67 (100)*	5.4 ± 5.1 (80)*	77 ± 54 (100)*	2.1 ± 3.8 (60)*
	Sotalol	78 ± 52 (100)	12 ± 12 (100)	44 ± 40 (100)	6.7 ± 7.1 (100)
Diuretics	Furosemide	26 ± 35 (50)*	236 ± 247 (100)*	46 ± 43 (100)*	214 ± 206 (100)*
	Hydrochlorothiazide	33 ± 52 (50)*	356 ± 373 (100)*	23 ± 35 (50)*	193 ± 218 (100)*
Lipid-regulators	Bezafibrate	0.8 ± 2 (25)	11.6 ± 14 (100)	0.7 ± 1 (25)*	4.1 ± 2.1 (100)*
	Fenofibrate	2623 ± 2020 (100)*	302 ± 232 (100)*	2678 ± 1863 (100)*	168 ± 189 (100)*
	Gemfibrozil	24 ± 29 (100)	91 ± 86 (100)	17 ± 25 (100)*	101 ± 88 (100)*
Psychiatric medications	Carbamazepine	15 ± 15 (100)	16 ± 19 (100)	13 ± 7 (100)	19 ± 18 (100)
	Diazepam	22 ± 24 (100)*	0.5 ± 0.7 (40)*	22 ± 25 (100)*	2.3 ± 4.1 (40)*
	Lorazepam	57 ± 40 (100)	53 ± 36 (100)	39 ± 8 (100)	71 ± 78 (100)
	Paroxetine	835 ± 1604 (100)*	26 ± 34 (80)*	3264 ± 4391 (100)	17 ± 33 (80)

Table 3. Daily mass load per 1000 inhabitants (ML, mg day^{-1}1000 inh^{-1}) of pharmaceutically active compounds (PhA group in the thickened sludge (ThS), acidogenic digester (AcD) effluent and methanogenic digester (MD) effluent of the pi digestion (MAD) during the experimental phases I and II.

Analytes	ThS		AcD		
	ML Phase I	**ML Phase II**	**ML Phase I**	**ML Phase II**	**ML P**
AIAPs	***26.5***	***146.0***	***32.3***	***91.7***	***28.3***
Acetaminophen	5.6	4.4	0.6	11.7	0.0
Codeine	1.1	6.3	0.8	4.3	0.3
Diclofenac	7.6	10.6	20.0	10.4	10.1
Ibuprofen	7.0	81.6	9.0	56.7	16.3
Indomethacin	0.1	0.3	0.1	0.2	0.1
Ketoprofen	1.5	10.5	1.2	5.3	0.9
Naproxen	3.0	32.4	0.5	3.2	0.1
Propyphenazone	0.6	0.1	0.3	0.0	0.5
Antibiotics	***293.1***	***34.2***	***1570.7***	***16.9***	***127.5***
Clarithromycin	1.8	0.6	0.5	0.3	0.2
Ofloxacin	283.7	30.4	1568.6	16.2	126
Sulfadiazine	1.8	0.0	0.4	0.0	0.2
Sulfamethazine	1.7	0.0	0.1	0.1	0.0
Sulfamethoxazole	3.1	1.4	1.1	0.0	0.3
Trimethoprim	1.0	1.8	0.1	0.4	0.0
Beta-blocker	***17.6***	***4.4***	***8.2***	***1.7***	***8.1***
Atenolol	1.3	3.2	0.9	1.3	0.3
Metoprolol	7.1	0.1	0.8	0.0	1.6
Propranolol	4.8	0.3	4.2	0.1	4.2
Sotalol	4.4	0.8	2.3	0.3	2.1
Diuretics	***3.0***	***37.8***	***3.4***	***19.5***	***1.9***
Furosemide	1.4	15.1	2.3	10.3	1.5
Hydrochlorothiazide	1.7	22.7	1.1	9.2	0.3
Lipid-regulators	***156.3***	***25.4***	***146.1***	***13.2***	***130.2***
Bezafibrate	0.0	0.7	0.0	0.2	0.0
Fenofibrate	155.0	18.9	145.2	8.0	129.2
Gemfibrozil	1.3	5.8	0.8	4.9	1.0
Psychiatric medications	***56.4***	***6.1***	***182.0***	***5.5***	***9.6***

Carbamazepine	0.8	1.0	0.7	0.9	1.0
Diazepam	1.2	0.0	1.2	0.1	1.4
Lorazepam	3.1	3.3	2.1	3.4	1.9
Paroxetine	51.3	1.6	178.0	1.0	5.3
TOTAL	***553.0***	***253.9***	***1942.6***	***148.5***	***305.6***
% AIAPs	4.8	58.0	1.7	62.0	9.3
% Antibiotics	53.0	13.0	81.0	11.0	42.0
% Beta-blocker	3.2	1.7	0.4	1.2	2.7
% Diuretics	0.6	15.0	0.2	13.0	0.6
% Lipid-regulators	28.0	10.0	7.5	8.9	43.0
% Psychiatric	10.0	2.0	9.0	4.0	3.0

3.3. Removal efficiencies (REs) of PhACs in the two-stage MAD plant

Table 4 shows the REs (mean + SD) calculated for the targeted PhACs in the AcD and MD, as well as the overall REs for the global two-stage MAD process, during the experimental phases I and II. Those compounds with average concentration values < 10 ng/g and a frequency of detection <50% in both phases (bezafibrate, indomethacin, propyphenazone, sulfadiazine and sulfamethazine, Table 2) were excluded from the calculations.

According to the Kruskal-Wallis test, statistically significant differences ($p < 0.05$ or $p < 0.10$) were observed between phases I and II for the REs of 2 PhACs in the AcD, 7 PhACs in the MD, and 4 PhACs in the global MAD process (Table 4). In phase I, the REs achieved for the majority of PhACs were similar in both digesters except for gemfibrozil, which showed a significantly higher RE in the AcD. In contrast, significantly higher REs of acetaminophen, atenolol, codeine, hydrochlorothiazide and lorazepam were measured in the MD compared to the AcD in phase II (REs > 70%, Table 4).

Mean RE values were statistically higher in the MD in phase II compared to phase I for carbamazepine (13 vs. -56 %), clarithromycin (88 vs. 39 %), codeine (91 vs. 70 %, respectively), gemfibrozil (16 vs. -141 %), ibuprofen (17 vs. -306 %), lorazepam (79 vs. 15%), and propranolol (100 vs. 18 %). The Spearman rank-correlation test (Table S4) showed that the SRT was the only operational/environmental variable displaying a strong correlation ($p < 0.01$) with the REs of the aforementioned PhACs in the MD, with the only exception of codeine and clarithromycin. Consequently, the extension of the SRT from 12 (phase I) to 24 days (phase II) was significantly connected with an improved removal of most PhACs in the MD.

The influence of SRT on the efficiency of removal of PhACs in anaerobic digesters has been seldom addressed in previous studies, leading to some contradictory conclusions. Samaras et al. (2014) and Gonzalez-Gil et al. (2016) reported increases of the mean REs of some organic micropollutants when the SRT was extended from 8 to 20 days and from 10 to 30 days, respectively, in line with the results presented here, while Carballa et al. (2007) and Yang et al. (2016) did not observed a significant influence of SRT on the REs of PhACs and personal care products.

Table 4. Mass removal efficiencies (REs, %) of the pharmaceutically active compounds (PhACs) in the acidogenic dige
and global two-stage mesophilic anaerobic digestion (MAD) process, measured in the experimental phases I (n=4) and
According to the Kruskal -Wallis test, values marked with asterisks ($p< 0.05$ * and $p<0.1$ **) were significantly differen
and II) in the same stage (AcD, MD or full MAD process), and values marked with ^{x} were significantly different ($p<0.05$
MD) in the same experimental phase (I or II).

		AcD		MD	
Therapeutic groups		**Phase I**	**Phase II**	**Phase I**	**Phase II**
AIAPs	**Acetaminophen**	90 ± 0	-8167 ± 16770^{x}	100 ± 0	98 ± 3.3^{x}
	Codeine	-41 ± 225	23 ± 26^{x}	70 ± 7.8*	91 ± 10^{*x}
	Diclofenac	-184 ± 319	-15 ± 41	8.4 ± 72	36 ± 4.3
	Ibuprofen	-294 ± 254	-5.8 ± 42	-306 ± 232*	17 ± 14*
	Ketoprofen	15 ± 35	16 ± 51	35 ± 60	38 ± 16
	Naproxen	90 ± 11	85 ± 5.2	93 ± 14	80 ± 17
Antibiotics	**Clarithromycin**	42 ± 97	68 ± 40	39 ± 32 **	88 ± 6.2**
	Ofloxacin	-390 ± 784	39 ± 28	64 ± 25	50 ± 12
	Sulfamethoxazole	67 ± 32	100 ± 0	60 ± 50	-
	Trimethoprim	85 ± 20	80 ± 6.9	90 ± 8.9	89 ± 15
Beta-blockers	**Atenolol**	31 ± 31	42 ± 27^{x}	72 ± 36	77 ± 25 ^{x}
	Metoprolol	90 ± 2.0	90 ± 14	-76 ± 185	100 ± 0
	Propranolol	20 ± 65	53 ± 86	18 ± 90**	100 ± 0**
	Sotalol	43 ± 27	36 ± 46	-60 ± 243	-2.2 ± 76
Diuretics	**Furosemide**	-26 ± 13	-2.9 ± 46	28 ± 59	35 ± 14
	Hydrochlorothiazide	34 ± 4.6	44 ± 23^{x}	82 ± 25	79 ± 5.6^{x}
Lipid-regulators	**Fenofibrate**	10 ± 37**	57 ± 29**	18 ± 83	69 ± 32
	Gemfibrozil	46 ± 30^{*x}	-20 ± 52*	-141 ± 160^{*x}	16 ± 29*
Psychiatric medications	**Carbamazepine**	-29 ± 72	-136 ± 260	-56 ± 60 *	13 ± 25*
	Diazepam	-36 ± 105	-	-69 ± 164	-
	Lorazepam	20 ± 39	-5.0 ± 66 ^{x}	15 ± 63 *	79 ± 13^{*x}
	Paroxetine	-2241 ± 4278	-242 ± 647	19 ± 106	-911 ± 1626

High mean REs (>80%) with low standard deviations (<10%) were achieved throughout the global two-stage MAD process at least in one of the experimental phases for acetaminophen, clarithromycin, hydrochlorothiazide, naproxen, sulfamethoxazole, and trimethoprim (Table 4). Similar REs have been found in the literature for these PhACs in sludge AD processes operating under different conditions (Gonzalez-Gil et al., 2016; Martín et al., 2012; Narumiya et al., 2013; Phan et al., 2018; Radjenović et al., 2009; Samaras et al., 2014, 2013; Yang et al., 2017, 2016), with the exception of hydrochlorothiazide, for which various authors (Jelić et al., 2012; Radjenović et al., 2009) have observed lower average RE values (71% and 55%, respectively). Very low (< 30%) or negative mean REs were measured for the global two-stage MAD process in both experimental phases for carbamazepine, diazepam, diclofenac, gemfibrozil, ibuprofen, and paroxetine (Table 4), in accordance with previous reports (Carballa et al., 2007b; Gonzalez-Gil et al., 2019; Narumiya et al., 2013; Phan et al., 2018; Radjenović et al., 2009; Yang et al., 2017, 2016), except for the AIAP ibuprofen, for which medium-to-high REs (ranging 30 to 95%) (Carballa et al., 2007b; Samaras et al., 2014, 2013) or very low REs (ranging from negative RE values to 20%) have been previously measured (Gonzalez-Gil et al., 2016; Phan et al., 2018; Radjenović et al., 2009; Yang et al., 2017, 2016).

Several authors have proposed that the molecular structure of trace organic compounds is the major factor governing their removal from wastewater (Phan et al., 2018; Tadkaew et al., 2011; Wijekoon et al., 2015; Yang et al., 2017, 2016). Hydrophobic PhACs or hydrophilic PhACs with strong electron-donating functional groups (EDGs) are more easily removed under anaerobic conditions, while those carrying at least one strong electron-withdrawing functional group (EWG) were more resistant to removal (Wijekoon et al., 2015; Yang et al., 2016).

In the present study, among those hydrophilic PhACs with strong EDGs previously referenced in literature (Wijekoon et al., 2015; Yang et al., 2016) only acetaminophen, atenolol, hydrochlorothiazide, naproxen, sulfamethoxazole and trimethoprim showed high REs (>70 %) in both experimental phases of the global two-phase MAD process (Table 4). Otherwise, clarithromycin, codeine, fenofibrate, metoprolol, and propranolol were highly removed (> 80%) (Table 4) only under the operating conditions fixed for the experimental phase II (SRTs of 5 and 24 days for AcD and MD, respectively. However, the mean REs for gemfibrozil were very low (0.1 and 4.6 % in phase I and II, respectively). Tadkaew et al. (2011) and Yang et al. (2016) classified this compound as a hydrophilic PhAC with EWG, and as such more resistant to anaerobic removal.

PhACs with EWGs like carbamazepine, diazepam, diclofenac and ibuprofen were not easily removed under the anaerobic conditions, in accordance with previous studies (Wijekoon et al., 2015; Yang et al., 2016), while others like ketoprofen, lorazepam and ofloxacin showed mean REs around 50% in at least one experimental phase of the global two-stage MAD process (Table 4). Even though ibuprofen and ketoprofen are hydrophilic PhACs (logD < 3.2) with strong EDGs (Tadkaew et al., 2011) and therefore likely to be removed under anaerobic conditions, observed RE values were only remarkable for ketoprofen (Table 4).

It is important to note that the highest REs for lorazepam and ofloxacin were achieved only under the operating conditions fixed for the experimental phase II, as it was remarked previously for several hydrophilic PhACs with strong EDGs (clarithromycin, codeine, fenofibrate, metoprolol, and propranolol). These results show that additional factors other than the chemical structure of PhACs (for example the operational/environmental conditions) could be decisive for their removal.

3.4. Quantification of Bacteria, Archaea and Fungi

Table S5 shows the absolute abundances of Bacteria, Archaea and Fungi in the AcD and MD effluent samples, measured by qPCR throughout the experimental phases I and II. The absolute abundances of Bacteria were in the range of 10^8-10^9 copies L^{-1} sludge in the AcD effluent, while they were kept around 10^{10} copies L^{-1} sludge in the MD effluent. The abundance of Archaea was *ca.* 10^8 copies L^{-1} sludge in the AcD effluent, and ranged between 10^8-10^9 copies L^{-1} sludge in the MD effluent. According to the Kruskal-Wallis and Conover-Iman tests, there were only significant differences of the numbers of both Bacteria and Archaea markers between the two experimental phases in the MD effluent samples (Table S5). The higher counts of Archaea in the MD in phase I agree with increased CH_4 recovery rates (Table 1). The average abundances observed here were below the ranges previously reported in different types of anaerobic digesters treating solid wastes, high-strength wastewaters, or municipal sewage sludge, measured by qPCR and digital droplet PCR approaches (10^{13}-10^{14} and 10^{10} -10^{13} copies of 16S rRNA genes of Bacteria and Archaea per L sludge, respectively; Jang et al., 2014; Kim et al., 2015; Nettmann et al., 2010).

Regarding fungal populations, amplification products could not be detected in samples of AcD and MD effluents in phase II, with any of the two primer pairs used, in agreement with the previous analyses conducted in the water line of the A^2O process

(Gallardo-Altamirano et al., 2018). In addition, quantifications based on the FungiQuant primer set, which was selected because of its higher specificity for fungi compared to other commonly used primers (Maza-Márquez et al., 2020), were below the limit of detection in several phase I samples. Nonetheless, average quantifications were 10^6-10^7 and 10^9 copies L^{-1} sludge for the FungiQuant and LR primers sets, respectively, indicating that, when detectable, fungal populations in the two-stage MAD represented an important fraction of the total microbial community.

3.5. Structure of bacterial, archaeal and fungal communities in the two-stage MAD plant

The diversity of Bacteria, Archaea and Fungi in the two-stage MAD plant was assessed by means of massive parallel sequencing of partial small-subunit rRNA genes. A total of 16 samples were analyzed (four effluent samples from each stage, AcD and MD, collected through each of the two experimental phases). Fungal diversity was not investigated in phase II samples, which failed to render any amplification products, in agreement with the results of the qPCR experiments. Following quality filtering of the raw sequencing data, 152052 and 261977 16S rRNA reads were kept for Bacteria and Archaea, respectively, and 18452 18S rRNA reads for Fungi. The number of sequencing reads per sample ranged 3998-19967 for Bacteria, 7653-50243 for Archaea, and 659-4171 for Fungi. In spite of these differences among the samples analyzed, the R/E curves (Figure S7) indicated that enough sequencing depth was achieved to appropriately describe the diversity of the three targeted microbial groups in the two-stage MAD system. Applying a 97% sequence identity cut-off, a total of 1341 Bacteria PHYs, 108 Archaea PHYs, and 246 Fungi PHYs were identified, with the numbers of PHYs per sample ranging 68-785, 10-70, and 51-126, respectively.

Table S6 summarizes the numbers of average reads of Bacteria, Archaea and Fungi per digester and experimental phase, as well as the average values of Richness (S), Shannon-Wiener (*H'*), Simpson's (1-D) and Chao-1 indices of diversity. For both Bacteria and Fungi, the indices described communities with medium-to-high species diversity and low functional organization. In comparison, the average values of *H'* and 1-D indices indicated a much lower diversity and community evenness of Archaea in both digesters, particularly in phase II samples. The diversity of the Bacteria communities was also lower in the samples retrieved throughout phase II, in both digesters.

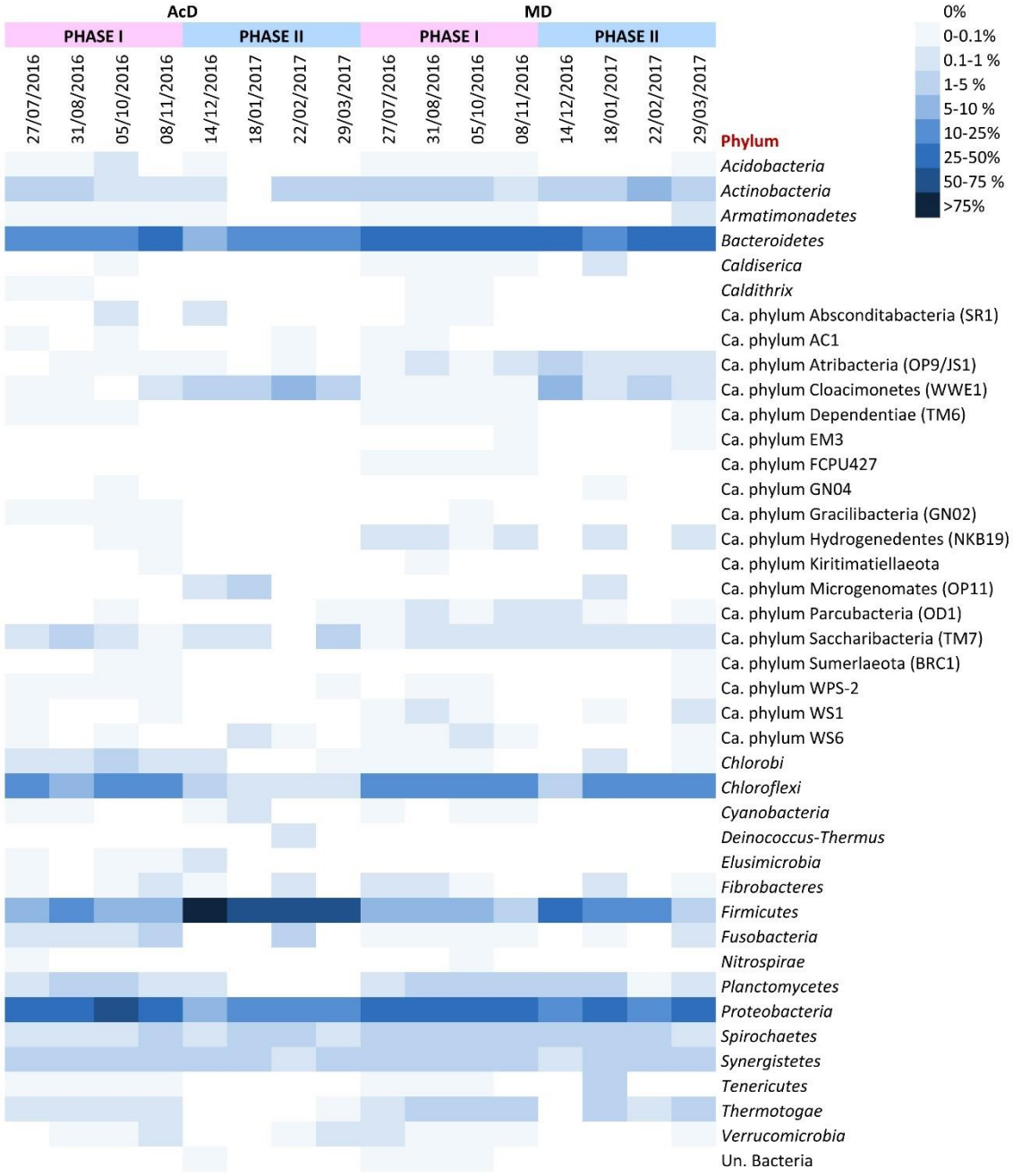
The dendrograms in Figure S8 show the clustering of the AcD and MD samples according to the dis(similarity) of their bacterial, archaeal and fungal communities (relative abundances of PHYs detected by Illumina sequencing). The global similarity of both Bacteria and Archaea communities among samples was < 20%; however, ANOSIM analyses showed that there were no significant differences among the community profiles of either Bacteria or Archaea in samples retrieved from the same digester ($R = 0.43$ and 0.81, respectively, $p < 0.05$) or throughout the same experimental phase ($R = 0.72$ and 0.63, respectively, $p < 0.05$).

3.5.1. Diversity of Bacteria communities

Taking the 16 samples altogether, Bacterial PHYs with > 0.1% average relative abundance were classified in 16 different Phyla, with *Proteobacteria*, *Bacteroidetes* and *Firmicutes* cumulatively comprising nearly 80% of the community (29.74, 25.18 and 24.02%, respectively), while 29 Phyla had a relative abundance > 0.1% in at least one sample (Figure 1). The average relative abundances of bacterial clades at the Order and Family levels in the global two-stage MAD process are summarized in Table S7.

The dominant Phyla within the bacterial communities varied depending on the digester and experimental phase. In the AcD effluent, *Proteobacteria* dominated in phase I samples (46.61% average relative abundance) followed by *Bacteroidetes*, *Chloroflexi* and *Firmicutes* (25.59, 11.36 and 7.66%, respectively), while *Firmicutes* were prevalent in phase II (62.05 % average relative abundance) followed by *Bacteroidetes* and *Proteobacteria* (12.85 and 12.54%, respectively). In the MD effluent, *Bacteroidetes* and *Proteobacteria* codominated in both experimental phases (38.26% and 31.23% in phase I, 24.02% and 28.60% in phase II, respectively), followed by *Chloroflexi* and *Firmicutes* (13.21% and 7.44% in phase I, 18.94% and 13.76 in phase II, respectively). The top four aforementioned bacterial phyla (*Bacteroidetes, Chloroflexi*, *Firmicutes,* and *Proteobacteria*) are often detected as major components of the bacterial community in mesophilic sewage sludge AD systems by metagenomic approaches based on high-throughput sequencing (McIlroy et al., 2017; Nelson et al., 2011; Wu et al., 2017; Yang et al., 2014), and comprise microorganisms that are known to participate in one or more of the stages of the AD process (hydrolysis, acidogenesis and acetogenesis) (Westerholm and Schnürer, 2019; Xia et al., 2016).

Figure 1. Heatmap displaying the relative abundances of Bacteria phylotypes (Phylum level), detected by Illumina sequencing in the acidogenic digestor (AcD) and the methanogenic digestor (MD) effluent samples in the experimental phases I and II.



Members of recently proposed Candidate Phyla were detected in most samples, some of which reached average relative abundances >1% within the bacterial communities. *Ca.* Saccharibacteria (TM7) were detected at relative abundances ranging 0.07-4.87% in the AcD effluent samples of phase I, while *Ca.* Cloacimonetes (WWE1) were particularly

abundant in phase II samples, in both the AcD and MD effluents (1.85-8.66% and 0.40-5.52%, respectively), and *Ca.* Atribacteria (OP9/JS1) were only detected in MD samples of phase II (0.15-4.32% relative abundance). The roles of these new Phyla in WWTPs or AD processes are just starting to be elucidated, but there are evidences of their involvement in organic matter depolymerization, fermentation, and syntrophic associations with methanogens (Kindaichi et al., 2016; Nobu et al., 2016; Westerholm et al., 2020).

Differences of the community composition among the two digesters and the experimental phases were also detected at the Class, Order and Family levels. *Bacteroidia*/*Bacteroidales* displayed the highest relative abundances in the AcD effluent in phase I samples and in the MD effluent in both experimental phases, while *Clostridia and Bacilli*/*Clostridiales* and *Turicibacterales* codominated the community in the AcD effluent in phase II samples (data not shown). Figure S9 displays a heatmap of the relative abundances of bacterial PHYs clustered at the Family level, including only those with ≥1% abundance in at least one sample. The most abundant families in the AcD effluent community were *Rikenellaceae* in phase I (11.13%) and *Turicibacteraceae* in phase II (25.00%), while *Rikenellaceae* were prevalent in the MD effluent in both experimental phases (33.8 9 in phase I and 18.33% in phase II).

SIMPER analyses were carried out aiming to evaluate which PHYs contributed most to the dis(similarity) of the bacterial communities in the different groups of samples analyzed (Table S8). The overall similarity was lower among the AcD effluent samples (28.09%) compared to the MD (39.95%). When samples were grouped by experimental phases, the overall similarity was higher in phase I (55.94%) than in phase II (25.73%). SIMPER results also allowed to conclude that the bacterial community was highly dissimilar when comparing among the two digesters (69.08%) or the two experimental phases (75.05%). Five PHYs (PHY0384, PHY0142, PHY0527, related to the families *Turicibacteraceae, Rikenellaceae* and *Peptostreptococcaceae*, respectively, and PHY0453 and PHY0321 both related to the *Clostridiaceae*) were among those making the highest contributions to explain such dissimilarities, either among the two digesters or the experimental phases; however, the contributions were always <2% for each individual PHY (Table S8E and F).

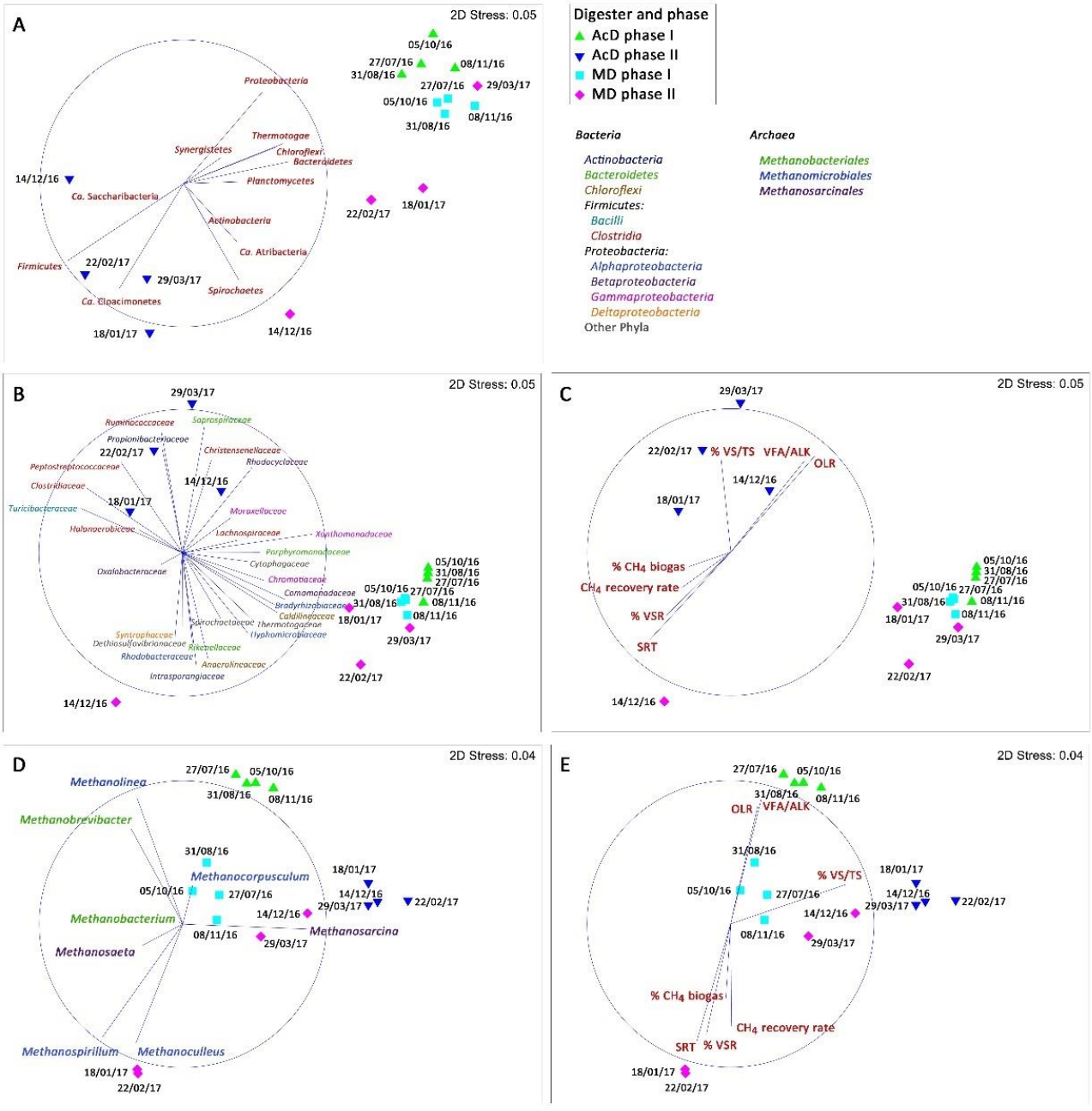
To better illustrate the observed differences of the bacterial community structure in the AcD and MD effluents during the two experimental phases, the samples were ordinated by MDS on the basis of the relative abundances of the PHYs identified by Illumina sequencing, clustered at the Phyla and Family levels (Figures 2A, 2B). At both taxonomic

levels, the ordinations show that the community structure was more similar between the two digesters in phase I than in phase II, as also observed with SIMPER analysis. Remarkably, a strong negative correlation occurred among *Proteobacteria* and *Firmicutes*, two of the most abundant Phyla in the samples (Figures 2A). *Comamonadaceae* and *Xanthomonadaceae* were the main families of the *Proteobacteria* increasing their relative abundances in the samples of the AcD during phase I, while *Clostridia* families (*Christensenellaceae*, *Clostridiaceae*, *Halanaerobiaceae*, *Peptostreptococcaceae*, *Ruminococcaceae*) together with *Turicibacteraceae* prevailed in the AcD throughout phase II. Samples of the MD had a more similar community in the two phases, sharing 7 of the 10 families with the highest relative abundances: *Anaerolinaceae*, *Caldilineaceae* (*Chloroflexi*), *Rikenellaceae* (*Bacteroidetes*), *Comamonadaceae*, *Hyphomicrobiaceae*, *Oxalobacteraceae*, and *Rhodobacteraceae* (*Proteobacteria*).

Overall, moderate-to-strong positive correlations (r = 0.5-0.75) were found among the indicators of the efficiency of methanogenesis (VSR, CH_4 in biogas, or CH_4 recovery rate) and the relative abundances of *Dethiosulfovibrionaceae*, *Rikenellaceae*, *Syntrophaceae*, and *Turicibacteraceae* (Figures 2B and C, Table S9). Contrariwise, the relative abundances of *Christensenellacae*, *Cytophagaceae*, *Lachnospiraceae*, *Moraxellaceae*, *Porphyrimonadaceae*, *Rhodocyclaceae*, and *Xanthomonadaceae* displayed strong negative correlations (r < -0.7) with VSR and CH_4 recovery rate. The abovementioned families plus *Moraxellaceae* and *Propionibacteriaceae* were correlated positively (r = 0.5-1.0) with the values of the VFA/ALK ratio and OLR (Figures 2B and C, Table S9).

In the present study, the dominant phylotypes in the AcD shifted from *Proteobacteria* in phase I to *Firmicutes* in phase II, showing that different groups of microorganisms acting as drivers of the hydrolysis and acidogenesis processes were selected under the different operating conditions applied. Among the families whose relative abundances displayed a positive correlation with the VFA/ALK ratio (Figures 2 and Table S9), *Cytophagaceae*, *Lachnospiraceae*, and *Xanthomonadaceae* were more abundant in phase I (Figure S9), when the AcD was subjected to the highest OLR, leading to a fast generation and accumulation of VFAs and low methane production (Table 1A), thus enabling an efficient separation of the AD process in two different stages. Members of the *Cytophagaceae* and *Lachnospiraceae* are both well-known for their key roles in the hydrolysis of several types of macromolecules, and *Lachnospiraceae* generate VFAs such as acetate, propionate and butyrate through fermentation (Biddle et al., 2013; McBride et al., 2014).

Figure 2. Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the samples retrieved from the acidogenic digestor (AcD) and the methanogenic digestor (MD) in phases I and II, according to the relative abundances of the Bacteria and Archaea phylotypes identified by Illumina sequencing. A. Ordination based on the relative abundance of Bacteria at the Phylum level. B. Ordination based on the relative abundance of Bacteria at the Family level. C. Correlations among operational parameters and the indicators of the efficiency of the AD process and the ordination shown in plot B. D. Ordination based on the relative abundance of Archaea at the Genus level. E. Correlations among the operational parameters and the indicators of the efficiency of the AD process and the ordination shown in plot D. Vectors in plots A., B. and E. represent the direction throughout the ordination of the relative abundances of the prokaryotic taxa with an average relative abundance ≥ 1% in a set of samples retrieved from the same digester and phase. Vectors in plots C. and E. represent the direction throughout the ordination of the abiotic variables volatile solids/total solids percentage (%VS/TS), volatile fatty acids to alkalinity ratio (VFA/ALK), sludge retention time (SRT), organic loading rate (OLR), and % of volatile solids removal (VSR). The stress level of the MDS plots (< 0.1) validates the 2D-representation of the biotic data distribution (Clarke and Warwick, 2001). Vectors with a length shorter than 0.2 had negligible influence on the ordination and are not shown.



On the other hand, *Xanthomonadaceae* are mostly strictly aerobic bacteria; however, they have been often found in significative relative abundances in AD bioreactors, being their roles in these systems still not well defined (Hao et al., 2016).

As previously stated, *Clostridiales* and *Turicibacterales* codominated the bacterial community in the AcD during phase II. Several families comprising well-characterized anaerobic acidogenic bacteria of the *Clostridiales* reached their highest relative abundances in the AcD (Figure S9), being consistently favored by higher %VS/TS (Figures 2 and Table S9B). Of these, only the *Christensenellaceae* and *Ruminococcaceae* were found positively correlated to the VFA/ALK ratio, together with *Propionibacteriaceae* (*Actinobacteria*) (Table S9B), which also occurred at high relative abundances in two samples of this experimental period (Figure S9). The *Turicibacteraceae* is a recently proposed family including only the Genus *Turicibacter*, described as anaerobic fermenting organisms generating lactate as the major end product (Bosshard et al., 2002; Verbarg et al., 2014). Although lactate is a common product of fermentation, its relevance as an intermediate of AD processes has been seldom addressed; however, it has been reported that lactate is efficiently oxidized to acetate in anaerobiosis during the acetogenic step, and the genes encoding this trait are widespread among Bacteria (Detman et al., 2018). Overall, *Clostridiceae* and *Halanaerobicaeae* together with *Turicibacteraceae* displayed positive correlations among them, and also with increasing %CH_4 in the biogas (Figure 2 and Table S9).

In the next step of the two-stage MAD, acetate, hydrogen and CO_2 are produced from VFAs in the MD prior to methanogenesis, through the activity of the homoacetogens and syntrophic acetogens which mostly belong to the *Firmicutes* and *Proteobacteria*, although members of the phyla *Actinobacteria*, *Bacteroidetes*, *Ca.* Cloacimonetes, *Chloroflexi*, and *Synergistetes* have been also postulated as partners or supporters of methanogens in syntrophic metabolic reactions (Narihiro et al., 2015; Westerholm and Schnürer, 2019). *Clostridiaceae*, *Halanaerobiaceae*, *Peptostreptococcaceae* and *Ruminococcaceae* (*Firmicutes*) were common to all samples of the two-stage MAD (Figure S9) and comprise bacteria able to provide methanogens with either hydrogen or acetate through homoacetogenesis (Drake et al., 2014; La Reau and Suen, 2018; Oren, 2014; Slobodkin, 2014); however, none of these families showed a strong positive correlation with CH_4 recovery rates (Table S9), suggesting that this step was carried out by a functionally redundant community and did not rely on the relative abundance of particular populations. In contrast, the relative abundances of the *Dethiosulfovibrionaceae* (*Synergistetes*),

Syntrophaceae (*Deltaproteobacteria*) and *Rikenellaceae* (*Bacteroidetes*) correlated positively with both VSR and CH_4 recovery rates, and at the same time were favored by longer SRTs (Figures 2B and Table S9). *Dethiosulfovibrionaceae* encompasses moderately halophilic anaerobic bacteria able to ferment peptides and amino acids generating substrates for methanogenesis such as acetate, H_2 and CO_2, among other end-products (Magot et al., 1997), and have been described as important players in the anaerobic treatment of fish-canning wastewaters (Militon et al., 2015). The Family *Syntrophaceae* were the only clade of well-known syntrophic acetogens reaching an average relative abundance >1% in the two-stage MAD, more specifically in samples of the MD during phase II. Finally, the species of the *Rikenellaceae* are regarded as anaerobic, mesophilic bacteria able to ferment carbohydrates or proteins, generating propionate, succinate, acetate, alcohols, H_2 or CO_2 as end products (Graf, 2014; Su et al., 2014). *Rikenellaceae* was shared by all samples (Figure S7) and was the most abundant taxa at the family level in the MD in both experimental phases, pointing to a key role of their interactions with methanogenic archaea for the performance of the two-stage MAD plant. Members of this clade have been found in high relative abundances in anaerobic WWTPs and AD reactors previously (Hao et al., 2016; Yi et al., 2014). *Rikenellaceae* relative abundance was negatively correlated with OLR, in agreement with previous reports (Hao et al., 2016).

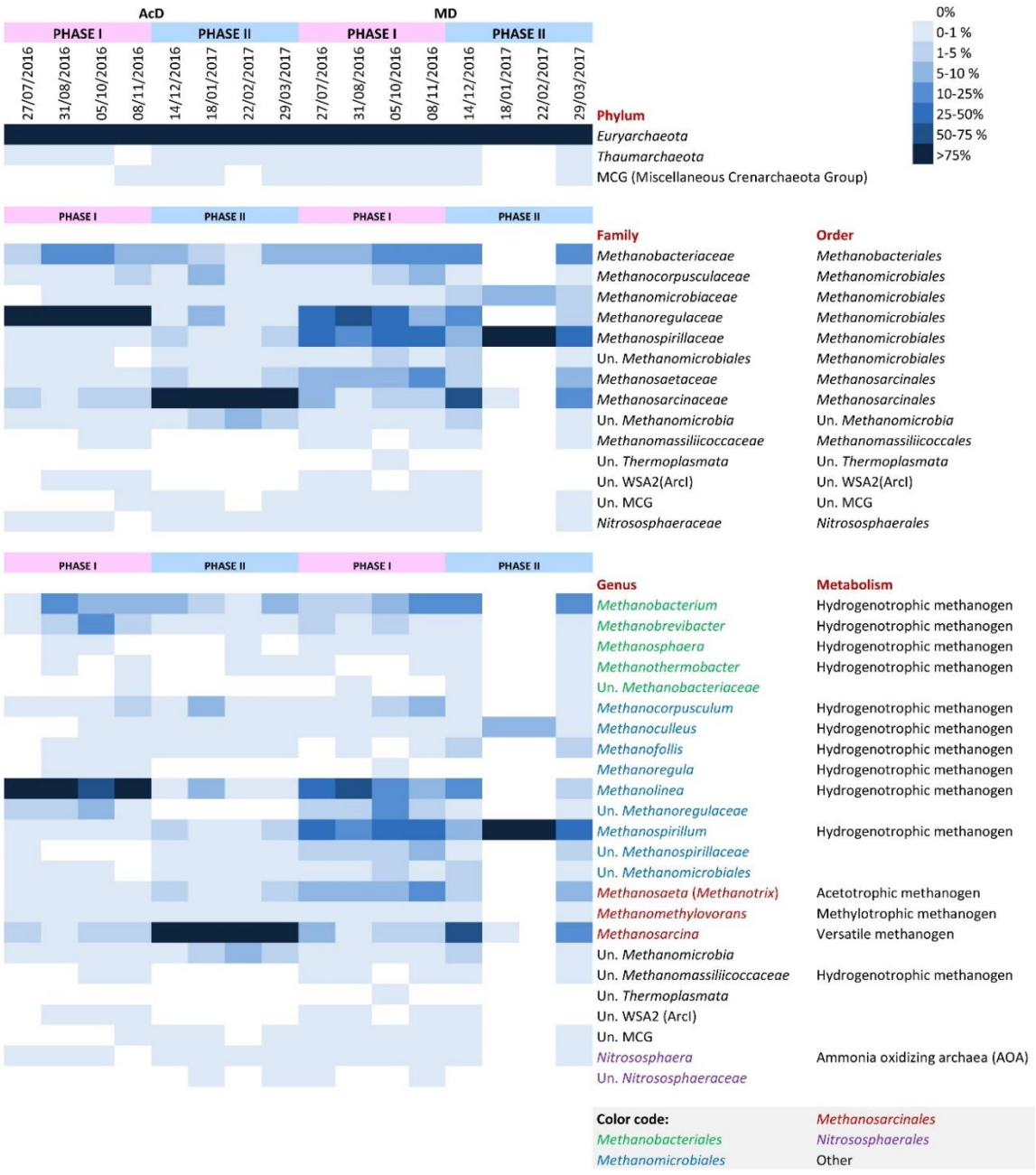
3.5.2. Diversity of Archaea communities

The heatmaps in Figure 3 display the relative abundances of archaeal PHYs in the two-stage MAD samples, at the Phylum, Family and Genus levels. On average, 99.90% of the Archaea characterized in the samples belonged to *Euryarchaeota*, while PHYs classified within the *Thaumarchaeota* and the miscellaneous *Crenarchaeota* group (MCG) were a minority. *Euryarchaeota* encompasses most of the methanogens, together with sulfate-reducing, extreme thermophilic and halophilic archaea (Oren, 2019).

Methanomicrobia were the dominant Class in either the AcD or the MD effluent samples, and throughout both experimental phases (75.00-100.00% relative abundance), followed by *Methanobacteria* (0.00-23.72%). *Methanomicrobiales* were also the prevalent Order in most samples, except those retrieved in phase II from the AcD effluent and one sample from the MD effluent, which were dominated by *Methanosarcinales* (59.73-92.10%). The analyses at the Family and Genus levels revealed that the prevalent members of the archaeal community differed among both the digesters and the experimental phases (Figure 3). The genera *Methanolinea* (*Methanoregulaceae*) (81.15%) and *Methanosarcina*

(*Methanosarcinaceae*) (86.99%) displayed the highest average relative abundances in the samples of the AcD effluent during phases I and II, respectively. In the MD effluent, the community was codominated by *Methanospirillum* (*Methanospirillaceae*) and *Methanolinea* in phase I (37.80 and 29.85%, respectively), while *Methanospirillum* prevailed in phase II (57.81%). The specialist acetoclastic *Methanosaeta* (*Methanotrix*), was only detected in higher relative abundances than *Methanosarcina* (6.96-18.25%) in the samples from the MD effluent in phase I

Figure 3. Heatmap displaying the relative abundances of Archaeal phylotypes (Phylum, Family and Genus levels), detected by Illumina sequencing in the acidogenic digestor (AcD) and the methanogenic digestor (MD) effluent samples in the experimental phases I and II.



According to SIMPER analysis (Table S10), the global similarities of the Archaea communities among samples of the AcD and MD effluents were 51.55% and 43.55%, respectively, while phase I and II samples displayed 56.59% and 40.45% similarities, respectively. As observed for Bacteria, the archaeal communities were highly dissimilar when compared either among the two digesters (68.56%) or the experimental phases (69.41%). PHY045 and PHY048 (*Methanolinea*), PHY061 (*Methanospirillum*), and PHY080 (*Methanosarcina*) were confirmed altogether as the major contributors to the observed dissimilarities, either among the digesters (37.77%) or the experimental phases (41.52%) (Table S10E and F).

Figure 2D displays the MDS ordination of the samples according to relative abundances of the archaeal PHYs identified by Illumina sequencing clustered at the Genus level, and their relationships with the shifts of the operational parameters and the indicators of the efficiency of methanogenesis are depicted in Figure 2E and Table S11. The relative abundances of *Methanospirillum* and *Methanoculleus* correlated strongly and positively with VSR, CH_4 recovery rates and $\%CH_4$ in the biogas ($r \geq 0.8$), while the prevalence of these two genera was favored by longer SRTs ($r \geq 0.9$) and lower ORL and VFA/ALK ratios ($r \leq -0.9$). In contrast, higher ORL and VFA/ALK ratios ($r \geq 0.7$) and shorter SRTs ($r \leq -0.7$) favored the dominance of *Methanolinea* and *Methanobrevibacter*, leading to a less efficient methanogenesis (Table S11).

Antagonistic trends were detected among the relative abundances of *Methanosaeta* and *Methanosarcina* throughout the experimental period (Figure 2D). Competition among these two genera has been thoroughly reported in previous studies (Castellano-Hinojosa et al., 2018) and is explained by differences in their respective affinities for acetate and utilization rates, which favor the growth of *Methanosaeta* in environments with low concentrations of acetate or VFAs, or low OLR (Xu et al., 2018; Yang et al., 2014). This is consistent with the correlations observed here among the relative abundances of *Methanosaeta* and the operational parameters %VS/TS, VFA/ALK ratio and OLR (r= -0.99, -0.66 and -0.65, respectively, Figure 2E, Table S11).

In the AcD, *Methanolinea* were prevalent in phase I, but were displaced by *Methanosarcina* in phase II (Figure 3), and this shift was connected with higher biogas and CH_4 production (Table 1A), making the separation of hydrolysis/acidogenesis from acetogenesis/methanogenesis only partial during phase II, as already addressed in section 3.1. In addition, Fountoulakis et al. (2004) revealed the inhibitory effect of some pharmaceuticals (i.e. diclofenac, carbamazepine or ofloxacin) on methanogens, and

Symsaris et al. (2015) showed that these negative effects were more evident on acetoclastic methanogens than on hydrogenotrophic methanogens. The high ML of ofloxacin detected in the AcD effluent in phase I (1568.6 mg day^{-1}1000 inh^{-1}, Table 3) could explain the underrepresentation of acetoclastic methanogens.

To sum up, the results of Illumina sequencing showed that methane generation in the two-stage MAD was mostly driven by the biological activity of carbon-dioxide reducing methanogens which use hydrogen or formate as primary electron donors (*Methanolinea*, *Methanospirillum*), or "versatile" methanogens such as *Methanosarcina,* able to generate CH_4 by acetoclastic, hydrogenoclastic and/or methylotrophic pathways (Oren, 2019). Steady-state in MAD processes has been classically connected to the prevalence of acetoclastic methanogens; however, stable operation has been also thoroughly reported in systems dominated by *Methanospirillum* (reviewed by Saha et al., 2020).

3.5.3. Diversity of Fungi communities

Figure S10 shows the heatmap of the relative abundances of fungal PHYs clustered at the Phylum and Order levels in both digesters of the two-stage MAD system during phase I. PHYs were affiliated to 7 different Phyla, and the community was dominated by *Ascomycota* in both AcD and MD effluent samples (87.69 and 83.92% average relative abundances, respectively), followed by *Basidiomycota* (7.70 and 10.63%, respectively), *Mortierellomycota* (4.29 and 3.66%), *Mucoromycota* (0.54 and 0.81%), *Chytridiomycota* (0.05 and 0.49%), *Blastocladiomycota* (0.12 and 0.43%), and *Kickxellomycota* (0.23 and 0.06%).

At the Order level, 41 different clades were detected, of which *Saccharomycetales* comprised nearly half of the identified reads (48.05%), and were followed in descending order of relative abundance by *Eurotiales*, *Hypocreales*, *Mortierellales*, *Trichosporonales* and *Microascales* (15.91, 6.47, 5.12, 3.93 and 3.76%, respectively). Comparing among the two digesters (Figure S10), *Saccharomycetales* displayed >55% relative abundance in all the samples of the AcD, while in the MD the fungal community was codominated by both *Saccharomycetales* and *Eurotiales* (35.03 and 21.85% average relative abundances, respectively). SIMPER analysis (Table S12) showed that the global similarities among the samples of the AcD and MD effluents were 42.54 and 45.37%, respectively. The fungal community was 61.89% dissimilar when comparing both digesters, and 15 PHYs mostly affiliated to the *Ascomycota* were required to explain > 25% of such dissimilarity. Overall, the structure and diversity of the fungal communities in the AcD and MD effluents were

similar to those previously characterized in the water line of the A^2O system during phase I (Gallardo-Altamirano et al., 2019), except for an increase of the relative abundance of *Ascomycota* and a reduction of the relative abundance of *Blastocladiomycota.*

Not many studies have been devoted to identify the fungal communities in anaerobic reactors, but those available demonstrate that fungi are viable and active in such environments and highlight relevant roles for these eukaryotic microorganisms in the hydrolysis of carbohydrates, lipids and proteins, and the generation of VFAs (Langer et al., 2019; Vinzelj et al., 2020). The dominance of *Ascomycota* and *Basidiomycota* in the fungal communities was previously observed in full-scale AD systems digesting or co-digesting feedstocks derived from plant, animal or food sources (Bücker et al., 2020; Langer et al., 2019; Young et al., 2018). Several genera of the *Ascomycota* are facultatively anaerobic yeasts with fermentative metabolism which display a variety of traits potentially contributing to the AD process, such as lipolytic and proteolytic activities and the ability to produce VFAs (Bücker et al., 2020).

Strictly anaerobic fungi of the *Neocallimastigomycota* were not detected in the two-stage MAD plant analyzed in this study, nor in the water line of the associated A^2O system (Gallardo-Altamirano et al., 2019). Members of this Phylum inhabit the gut of herbivores and establish mutualistic symbiosis with methanogens, enhancing the digestion of plant material (Hooker et al., 2019). These interactions are postulated to occur also in engineered AD processes, since *Neocallimastigomycota* have been detected by cultivation-independent methods in biogas reactors fed with mixtures of lignocellulose-rich plant materials and animal wastes (Dollhofer et al., 2017; Kazda et al., 2014). However, absence of strictly anaerobic fungi was reported in analogous AD systems (Langer et al., 2019; Young et al., 2018), in agreement with the results presented here. Besides the nature of the feedstock, several other factors are reported to strongly influence the occurrence and activity of *Neocallimastigomycota* in biogas reactors, including temperature and SRT (Vinzelj et al., 2020).

3.6. Linking population dynamics of Bacteria and Archaea to the REs of PhACs in the two-stage MAD plant

Figure 4 shows the MDS ordination of AcD and MD samples based on the REs of 6 PhACs which were effectively removed in both digesters in the two experimental phases. Vectors representing the trends through the ordination of either the REs (Figure 4A) or the relative abundances of bacterial (Figure 4B, C) and archaeal PHYs (Figure 4D) were

overlapped on the plots, and the Pearson's product-moment correlation coefficients among the vectors are displayed in Table S13. Only strong positive correlations ($r > 0.75$) among REs and the relative abundances of bacterial and archaeal groups will be discussed here.

The REs of naproxen and ketoprofen correlated strongly with the relative abundances of several families of *Firmicutes* of the class *Clostridia*, plus the families *Propionibacteriaceae* (*Actinobacteria*) and *Cytophagaceae* (*Bacteroidetes*), and the versatile methanogenic genus *Methanosarcina*. Similar trends were observed for trimethoprim, although the correlations were weaker ($r < 0.75$) for some of the abovementioned groups. The REs of ofloxacin and fenofibrate displayed strong correlations with the relative abundances of 8 bacterial families each, which belonged to the *Bacteroidetes*, *Beta-* and *Gammaproteobacteria* in the case of ofloxacin, and to the *Chloroflexi*, *Alpha-* and *Betaproteobacteria*, *Synergistetes* and *Thermotoga* in the case of fenofibrate. Strong correlations were also found among ofloxacin REs and the relative abundances of 4 genera of methanogens, including the acetoclastic specialist *Methanosaeta*, while the REs of fenofibrate correlated with the relative abundances of *Methanobrevibacter* and *Methanocorpusculum*. Finally, the REs of atenolol increased concomitantly with the relative abundances of three bacterial families (*Intrasporangiaceae*, *Oxalobacteraceae*, and *Syntrophaceae*; *Actinobacteria*, *Alpha-* and *Deltaproteobacteria*, respectively) plus the Candidate phyla Atribacteria and Cloacimonetes, and two genera of hydrogenotrophic methanogens (*Methanoculleus* and *Methanospirillum*).

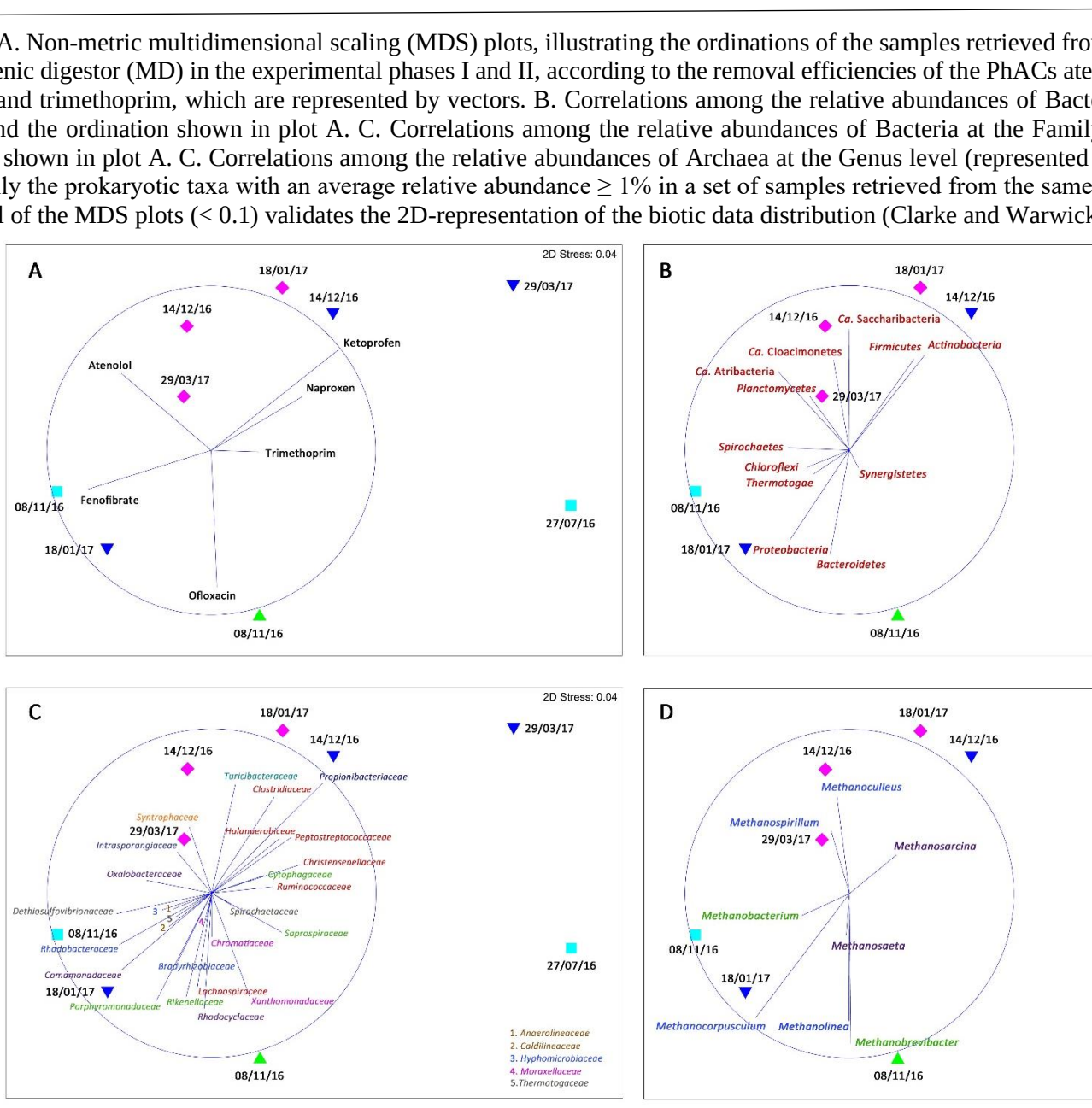
According to the literature, atenolol, naproxen, and trimethoprim are usually removed very efficiently (> 70%) in anaerobic WWT systems, while ofloxacin and ketoprofen are 30-70% removed (Lim et al., 2020), and few data are available for fenofibrate (Kujawa-Roeleveld et al., 2008). The biotransformation or biodegradation pathways followed by these and other PhACs in AD sludge remain largely unexplored to date; however, some findings reported in earlier studies which are in accordance with the observations presented in this work will be discussed below.

The biotransformation of naproxen and trimethoprim under anaerobic conditions is well characterized and takes place via *O*-demethylation of phenyl-methyl-ether groups (Ghattas et al., 2017). Wolfson et al. (2019, 2018) characterized the diversity of two consortia able to *O*-demethylate naproxen under methanogenic and sulfate-reducing conditions, observing an enrichment of fermentative and acetogenic bacteria, mostly belonging to the Phylum *Bacteroidetes* and/or the Class *Clostridia*; in addition, these

authors postulated that both acetoclastic and hydrogenotrophic methanogens could contribute to convert the methyl groups of naproxen into methane, either by readily using acetate or through an association with syntrophic acetate oxidizers, respectively. Interestingly, no overlap of the bacterial community structure was found among the two consortia, since the dominating families differed depending on the source of the inoculum (AD sludge or anoxic marine sediments), supporting that biotransformation of naproxen should rely on functionally redundant taxa. Similarly, Liang et al. (2019) reported the biotransformation of trimethoprim under sulfate-reducing conditions through *O*-demethylation and further degradation of the hydroxylated derivate, by consortia dominated (>60% cumulative abundance) by Classes *Bacteroidia*, *Sphingobacteriia*, (*Bacteroidetes*), *Clostridia* (*Firmicutes*), and *Deltaproteobacteria* (*Proteobacteria*). The positive correlations found here among the REs of naproxen and trimethoprim and the relative abundances of *Cytophagaceae* (*Bacteroidetes*), the acetogens of the Class *Clostridia* (ie. *Christensenellaceae*, *Halanaerobiaceae*, *Peptostreptococcaceae*, *Ruminococcacee*) and the Genus *Methanosarcina* (Table S13) are in accordance with the aforementioned studies and suggest that analogous patterns of biotransformation could take place in the two-stage MAD, although confirming this hypothesis will require additional research efforts.

Fluoroquinolone antibiotics tend to adsorb to sludge in WWTPs, hence reaching significant concentrations in AD systems, and are regarded as not easily biodegradable (Rusch et al., 2019). Nonetheless, these PhACs undergo a variety of biological transformations in the environment, mediated by both fungi and bacteria. Over 20 transformation products have been identified to date for ciprofloxacin, either generated by modification reactions (ie., acetylation, formylation, nitridation, or succinylation); cleavage of the piperazine ring; or through the decarboxylation, defluorination, or hydroxylation of the fluoroquinolone core structure (Rusch et al., 2019). Six of the characterized transformation products were identified in a lab-scale sulfate-reducing up-flow sludge bed reactor, and transformations involving hydroxylation and cleavage of the piperazine ring were postulated (Jia et al., 2019). It is assumed that, due to the large number of possible reactions, consortia of mixed species are more likely to be involved with biodegradation of fluoroquinolones in the environment (Rusch et al., 2019). Accordingly, in the present study, ofloxacin displayed strong positive correlations with a large number of evolutionarily dispersed bacterial families (Figure 4, Table S13).

Figure 4. A. Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the samples retrieved fro methanogenic digestor (MD) in the experimental phases I and II, according to the removal efficiencies of the PhACs ate ofloxacin and trimethoprim, which are represented by vectors. B. Correlations among the relative abundances of Bact vectors) and the ordination shown in plot A. C. Correlations among the relative abundances of Bacteria at the Famil ordination shown in plot A. C. Correlations among the relative abundances of Archaea at the Genus level (represented plot A. Only the prokaryotic taxa with an average relative abundance ≥ 1% in a set of samples retrieved from the same stress level of the MDS plots (< 0.1) validates the 2D-representation of the biotic data distribution (Clarke and Warwick



Fenofibrate is a highly hydrophobic compound which mostly adsorbs onto sludge of WWT systems (Faria et al., 2020). To the best of the authors' knowledge, no published studies are available concerning neither the possible biodegradation/biotransformation pathways nor the resulting metabolites of fenofibrate and the structurally-related compounds bezafibrate and gemfibrozil under anaerobic conditions. Since fenofibrate is an ester and bezafibrate an amide, their structures are prone to hydrolysis reactions (de Oliveira et al., 2016), and indeed, biodegradation of bezafibrate to 4-chlorobenzoic acid through hydrolysis of the amide bond was reported by Quintana et al. (2005) in a membrane bioreactor system operated aerobically. Interestingly, in this study the relative abundance of the *Rhodobacteraceae* showed a strong positive correlation with the REs of fenofibrate ($r = 0.98$), and similar patterns were previously observed for bezafibrate and gemfibrozil in the water line of the A^2O system associated to the two stage-MAD (Gallardo-Altamirano et al., 2019). The Family *Rhodobacteraceae* comprises phenotypically, metabolically and ecologically diverse species, widely distributed in aquatic environments, including WWTPs (Pujalte et al., 2014). Species of the *Rhodobacteraceae* often carry genes for the aerobic and anaerobic degradation of aromatic compounds (Carmona et al., 2009; Pérez-Pantoja et al., 2016); however, the possible involvement of these bacteria in the degradation of PhACs in AD systems has not been explored.

4. Conclusions

The results presented here provide new insights about the mass balance and REs of 27 PhACs in a two-stage MAD plant treating sewage sludge, also revealing robust interlinkages among operational parameters, performance of methanogenesis, shifts of the microbial communities' structure, and REs of PhACs. From the operational/performance point of view, it is concluded that an SRT of ≤ 2 days in the AcD is required to ensure effective separation of the acidogenesis and methanogenesis processes in two-stage MAD systems treating sewage sludge with %TS < 3%. SRT was also the most significant operational parameter influencing PhACs removal; in particular, the longer SRT used in the MD (24 d) significantly improved the REs of carbamazepine, clarithromycin, gemfibrozil, ibuprofen, lorazepam and propranolol. Besides SRT, ORL and VFA/ALK were also linked to shifts of the Bacteria and Archaea communities' structure in the AcD and MD sludges, which were subsequently correlated with differences in the efficiency of methanogenesis (VSR, CH_4 in biogas, and CH_4 recovery rate) among the experimental phases. Robust correlations were also detected among the REs of atenolol, fenofibrate, ketoprofen, naproxen, ofloxacin, and trimethoprim, and the relative abundances of

several bacterial and archaeal groups, pointing out to their potential involvement in the biodegradation/biotransformation of these xenobiotics under AD conditions.

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Supplementary material for Chapter 3:

"Insights into the removal of pharmaceutically active compounds from sewage sludge by two-stage mesophilic anaerobic digestion."

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Table S1. Optimized parameters for analysis of target analytes: target analyte, surrogate standard, abbreviation, retention transitions obtained in positive electrospray ionization by UPLC-HR-QTOF-MS.

Target compound	Abbrev.[a]			MRM-HR transitions	
		RT[b] (min)	Precursor ion (m/z)	Quantify ion	Qualify ion
Atenolol	ATN	2.65	267.1703	190.094	145.0673
Atenolol d7	ATN-d7	2.64	268.1907	190.0914	
Carbamazepine	CBZ	7.30	237.1022	194.1101	193.0934
Carbamazepine d10	CBZ-d10	7.24	247.1650	204.1622	
Clarithromycin*	CLA	8.09	748.4841	158.1272	590.4206
Codeine	COD	3.31	300.1594	152.0594	115.052
Codeine d3	COD-d3	3.30	303.1782	152.0599	
Diazepam	DZM	9.55	285.0789	154.0456	193.0918
Diazepam d5	DZM-d5	9.55	290.1103	198.1203	
Fenofibrate	FEN	13.67	361.1201	139.0002	233.0623
Fenofibrate d6	FEN-d5	13.64	367.1577	234.0547	
Lorazepam	LZM	7.90	321.0192	277.0186	305.0209
Lorazepam d4	LZM-d4	7.87	325.0443	279.0457	
Metoprolol	MTL	5.01	268.1907	116.1077	74.0597
Metoprolol d7	MTL-d7	4.99	275.2346	158.9905	
Ofloxacin*	OFX	4.23	362.1510	318.1679	261.1053
Paroxetine	PXT	7.52	330.1500	192.1302	70.0662
Paroxetine d4	PRX-d4	7.51	334.1751	74.0897	
Propranolol	PPL	6.46	260.1645	116.109	183.0843
Propranolol d7	PPL-d7	6.46	267.2084	189.1444	
Propyphenazone*	PPZ	7.82	231.149	56.0486	189.1047
Sotalol	STL	2.65	273.1267	213.0768	255.1307
Sotalol d6	STL-d6	2.63	279.1644	261.166	
Sulfadiazine	SDZ	3.30	251.0597	156.0318	92.0542
Sulfadiazine d4	SDZ-d4	3.28	255.0848	160.0419	
Sulfamethazine	SMZ	4.54	279.0910	186.0528	124.0937
Sulfamethazine d4	SMZ-d4	4.52	283.1161	186.044	
Sulfamethoxazole	SMX	5.59	254.0594	156.0312	92.0539
Sulfamethoxazole d4	SMX-d4	5.57	258.0845	160.0443	
Trimethoprim*	TMP	4.04	291.1451	230.1206	261.1026

[a] Abbreviation, [b] Retention time, [c] Accumulation time, [d] Declustering potential, [e] Collision energy *Compound quantifie

Table S2. Optimized parameters for analysis of target analytes: target analyte, surrogate standard, abbreviation, retention transitions obtained in negative electrospray ionization by UPLC-HR-QTOF-MS.

Target compound	Abbrev.[a]			MRM-HR transitions	
		RT[b] (min)	Precursor ion (m/z)	Quantify ion	Qualif
Acetaminophen	ATM	2.61	150.056	107.0379	118.0
Acetaminophen d4	ATM-d4	2.60	154.0811	121.0299	
Ibuprofen	IBU	7.16	205.1234	161.1341	
Ibuprofen d3	IBU-d3	7.16	208.1422	164.1528	
Gemfibrozil	GFZ	9.40	249.1496	121.0671	127.0
Gemfibrozil d6	GFZ-d6	9.42	255.1873	121.0661	
Diclofenac	DCF	7.14	294.0094	250.0205	214.0
Diclofenac d4	DCF-d4	7.13	298.0345	254.0453	
Furosemide	FSM	5.31	329.0004	285.0115	204.9
Furosemide d5	FSM-d5	5.29	334.0318	290.0425	
Indomethacin	IMT	7.32	356.0695	312.0798	297.0
Indomethacin d4	IMT-d4	7.32	360.0946	316.1056	
Bezafibrate	BFT	6.16	360.1008	274.0648	154.0
Bezafibrate d4	BFT-d4	6.16	364.1259	278.0892	
Ketoprofen	KTP	5.71	256.1058	209.0984	197.0
Ketoprofen C13d3	KTP-C13d3	5.71	257.1086	213.1209	
Naproxen	NPX	5.57	229.087	169.0665	185.0
Naproxen d3	NPX-d3	5.57	232.1058	173.0929	
Hydrochlorothiazide	HCT	3.52	295.9572	268.9476	204.9
Hydrochlorothiazide d2	HCT-d2	3.51	297.9697	269.9532	

[a] Abbreviation, [b] Retention time, [c] Accumulation time, [d] Declustering potential, [e] Collision energy

Table S3. Concentration range (ng/g) of pharmaceutically active compounds (PhACs) in the thickened sludge influent (Th
methanogenic digester (MD) effluent for the experimental phases I (n=4) and II (n=5).

Analytes	Phase I			Phase II	
	ThS	AcD	MD	ThS	A
AIAPs					
Acetaminophen	0 - 444	0 - 50	0 - 0	1 - 270	98
Codeine	6 -38	0 - 32	0 -14	24 - 245	19
Diclofenac	92 - 199	108 - 1110	108 - 471	64 - 395	90
Ibuprofen	8 - 518	43 - 584	301 - 752	445 - 3830	55
Indomethacin	1 - 3	2 - 3	1 - 3	0 - 9	1
Ketoprofen	7 - 81	7 - 69	0 - 89	44 - 416	52
Naproxen	17 - 144	0.2 - 39	0 - 17	108 - 1641	17
Propyphenazone	0 - 39	0 - 10	0.1 - 29	0 - 2	0
Antibiotics					
Clarithromycin	9 - 76	0 - 21	0 - 40	0 - 20	0
Ofloxacin	238 - 10800	69 - 122000	35 -7930	134 - 1032	11
Sulfadiazine	0 - 76	0 - 17	0 - 25	0 - 0	0
Sulfamethazine	0 - 64	0 - 3	0 - 1	0 - 3	0
Sulfamethoxazole	19 - 107	0 - 46	0 - 20	0 - 71	0
Trimethoprim	8 - 32	0 - 5	0 - 1	16 - 35	3
Beta-blockers					
Atenolol	20 - 27	6 - 23	0 - 19	10 - 133	8
Metoprolol	0 - 178	2 - 21	0 - 70	0 - 6	0
Propranolol	15 - 176	1 - 128	0 - 227	0 - 13	0
Sotalol	4 - 126	3 - 87	2 - 113	2 - 27	2
Diuretics					
Furosemide	0 - 75	6 - 107	0 - 93	65- 617	88
Hydrochlorothiaide	0 - 110	0 - 74	0 - 38	85 - 926	57
Lipid-regulators					
Bezafibrate	0 - 3	0 - 3	0 - 1.5	2 - 36	2
Fenofibrate	344 - 5210	172 - 4670	35 - 23900	92 - 500	33
Gemfibrozil	9 - 67	3 - 5	12 - 68	25 - 217	38
Psychiatrics					
Carbamazepine	3 - 36	5 - 21	11 - 41	1 - 46	6
Diazepam	1 - 56	1 - 58	1 - 84	0 - 2	0
Lorazepam	33 - 116	28 - 47	19 -92	25 - 112	22
Paroxetine	11 - 3240	1 - 9380	31 - 356	0 - 77	0

Table S4. Spearman rank-correlation coefficients (ρ) between the enviromental/operational variables of the methanogenic
(REs, %). Abiotic variables: ratio volatile free acid/alkalinity (VFA/ALK), volatile solids removal (VSR), organic loadin
Significant correlations are boldfaced in green and red (red: p<0.05; green: p < 0.10).

PhACs	pH	VFA/ALK	VSR	Biogas production	CH_4 production	Biogas recovery rate	CH_4 recovery rate
Acetaminophen	0.10	0.18	**0.78**	0.54	0.71	0.67	0.73
Codeine	0.11	-0.90	0.31	0.25	0.31	0.16	0.26
Diclofenac	0.20	-0.46	**0.70**	0.13	0.07	0.02	-0.34
Ibuprofen	0.38	**-0.62**	0.45	0.52	**0.65**	-0.08	-0.37
Ketoprofen	0.55	0.56	-0.55	-0.32	-0.38	-0.31	-0.34
Naproxen	-0.41	0.26	-0.47	-0.42	-0.36	0.04	**0.67**
Clarithromycin	0.70	-0.79	0.50	0.50	0.10	-0.30	**-0.90**
Ofloxacin	-0.55	0.27	-0.25	**-0.58**	-0.52	-0.29	0.03
Sulfamethoxazole	-	-	-	-	-	-	-
Trimethoprim	-0.48	-0.35	0.06	-0.16	-0.16	-0.18	0.26
Atenolol	-0.39	-0.52	**0.71**	0.49	0.39	0.57	0.24
Metoprolol	-0.10	-0.68	0.41	0.56	0.56	-0.41	-0.68
Propranolol	0.37	**-0.80**	**0.65**	**0.75**	0.57	-0.24	**-0.72**
Sotalol	-0.32	-0.20	**0.62**	0.38	0.25	**0.83**	0.37
Furosemide	0.12	-0.29	**0.68**	0.08	-0.04	0.07	-0.38
Hydrochlorothiazide	-0.07	-0.45	0.07	-0.32	-0.29	0.02	0.41
Fenofibrate	-0.13	**-0.67**	0.40	0.53	**0.60**	0.13	-0.14
Gemfibrozil	0.22	-0.48	0.17	0.00	0.03	-0.34	**-0.66**
Carbamazepine	**0.77**	-0.32	0.38	0.17	0.03	-0.46	**-0.92**
Lorazepam	0.33	**-0.87**	0.37	0.37	0.47	-0.38	-0.41
Paroxetine	0.11	0.09	-0.04	0.21	0.36	0.29	0.64

Table S5. Number of copies per L of sludge (means and standard deviations) of gene markers of Bacteria, Archaea and Fu
and methanogenic effluents (AcD and MD, respectively) of the two-stage MAD plant, in the experimental phases I and II
set of primers: FungiQuant and LR. n.d: amplification not detected. Values marked with the same letter in each column are
Conover-Iman tests, $p < 0.05$).

Phase	Stage	Bacteria	Archaea	Fungi (FungiQuant)
I	**AcD**	$3.92\ 10^{9} \pm 7.44\ 10^{9}$ (a)	$6.51\ 10^{8} \pm 9.77\ 10^{8}$ (a)	$6.66\ 10^{7} \pm 8.96\ 10^{7}$ * (a)
	MD	$1.04\ 10^{10} \pm 6.95\ 10^{9}$ (b)	$6.88\ 10^{9} \pm 5.32\ 10^{9}$ (b)	$7.42\ 10^{6} \pm 5.20\ 10^{6}$ ** (a
II	**AcD**	$5.67\ 10^{8} \pm 1.01\ 10^{8}$ (ab)	$1.33\ 10^{8} \pm 4.51\ 10^{7}$ (a)	n. d
	MD	$6.26\ 10^{10} \pm 6.74\ 10^{10}$ (c)	$3.80\ 10^{8} \pm 4.42\ 10^{8}$ (a)	n. d

* 50% of samples rendered values below the limit of detection and were not considered for calculations
** 25% of samples rendered values below the limit of detection and were not considered for the calculations

Table S6. Number of average reads per sample, average phylotypes (PHYs) richness (S), and average plus standard deviatio
Wiener (*H'*) and Simpson's (1-D) evenness, calculated from the Illumina sequencing data of Bacteria, Archaea and Fun
(AcD) and methanogenic digester (MD) effluent samples, in experimental phases I and II.

Target	Phase	Average reads per sample	Average Richness (S)	Chao-1 richness estimation	Shanno Wiener
AcD effluent					
Bacteria	**I**	8218.8 ± 3314.1	553.3 ± 110.8	732.2 ± 125.9	4.54 ±
	II	9904.3 ± 5365.0	160.5 ± 101.8	196.2 ± 122.7	3.13 ±
Archaea	**I**	10237.5 ± 1489.3	37.8 ± 6.3	43.9 ± 10.9	1.41± 0
	II	10896.8 ± 2637.8	38.3 ± 4.2	49.4 ± 11.4	0.85 ±
Fungi	**I**	1509.3 ± 1189.5	72.3 ± 17.4	106.0 ± 23.3	3.05 ±
MD effluent					
Bacteria	**I**	14300.3 ± 7239.3	633.0 ± 178.8	820.9 ± 195.9	3.92± 0
	II	5589.8 ± 1489.7	203.8 ± 120.1	264.0 ± 172.0	3.65 ±
Archaea	**I**	31168.3 ± 17837.6	62.8 ± 3.3	72.4 ± 5.1	2.38 ±
	II	13191.8 ± 5122.1	36.8 ± 30.8	39.5 ± 33.1	1.33 ±
Fungi	**I**	3103.8 ± 1037.4	106.3 ± 15.8	125.0 ± 19.7	3.40 ±

Table S7. Relative abundances of bacterial Orders and Families in the global two-stage MAD process (average % ± standard deviation of samples from both the acidogenic digester (AcD) and methanogenic digester (MD) effluent samples, throughout experimental phases I and II). Taxa with average relative abundances <1% are not shown. Un.: unclassified

Taxa	average % ± SD	Taxa	average % ± SD
Bacteroidales	**22.49 ± 11.65**	***Rhodobacterales***	**4.62 ± 3.66**
Porphyromonadaceae	2.75 ± 2.44	*Rhodobacteraceae*	4.59 ± 3.67
Rikenellaceae	17.26 ± 12.17	***Xanthomonadales***	**4.31 ± 3.82**
Un. *Bacteroidales*	1.69 ± 1.43	*Xanthomonadaceae*	4.07 ± 3.68
Clostridiales	**13.46 ± 13.40**	***Anaerolineales***	**3.03 ± 3.00**
Clostridiaceae	5.88 ± 7.14	*Anaerolinaceae*	3.03 ± 3.00
Lachnospiraceae	1.18 ± 1.54	***Synergistales***	**2.38 ± 1.16**
Peptostreptococcaceae	3.00 ± 4.78	*Dethiosulfovibrionaceae*	1.23 ± 0.81
Turicibacterales	**8.45 ± 12.95**	***Chromatiales***	**2.30 ± 2.49**
Turicibacteraceae	8.45 ± 12.95	*Chromatiaceae*	2.28 ± 2.48
Caldilineales	**6.30 ± 5.00**	**Un. Ca. phylum Cloacimonetes**	**1.68 ± 2.56**
Caldilineaceae	6.30 ± 5.00	***Rhodocyclales***	**1.59 ± 0.92**
Burkholderiales	**6.20 ± 4.06**	*Rhodocyclaceae*	1.59 ± 0.92
Comamonadaceae	3.46 ± 3.50	***Cytophagales***	**1.45 ± 1.88**
Oxalobacteraceae	2.35 ± 1.52	*Cytophagaceae*	1.45 ± 1.88
Rhizobiales	**4.77 ± 3.27**	***Actinomycetales***	**1.45 ± 1.22**
Hyphomicrobiaceae	2.29 ± 1.79	***Halanaerobiales***	**1.37 ± 1.96**
		Halanaerobiaceae	1.37 ± 1.96

Table S8. Phylotypes (PHYs) which mostly contributed to the global similarity of the bacterial communities between (A) samples of the acidogenic digester (AcD) effluent, (B) samples of the methanogenic digester (MD) effluent, (C) samples of both digesters effluents in phase I (C), and (D) samples of both digesters effluents in phase II (D), according to SIMPER analysis. (E) PHYs which mostly contribute to the dissimilarity of the bacterial communities between samples of the AcD and MD effluents (F) PHYs which mostly contributed to the dissimilarity of the bacterial communities of both digester effluents between samples of phases I and II, according to SIMPER analysis.

A.

Average similarity among samples of the AcD effluent: 28.09%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY0142	*Rikenellaceae*	22.11	6.76	6.76
PHY0384	*Turicibacteraceae*	23.98	4.60	11.36
PHY0527	*Peptostreptococcaceae*	15.73	2.71	14.06
PHY0407	*Clostridiaceae*	9.94	2.10	16.17
PHY0453	*Clostridiaceae*	14.14	2.10	18.27
PHY1259	*Xanthomonadaceae*	10.45	2.08	20.35
PHY1009	*Comamonadaceae*	11.37	2.01	22.36
PHY0245	Unclassified Candidate phylum Cloacimonetes (WWE1)	8.89	1.86	24.22
PHY0648	*Halanaerobiaceae*	8.63	1.64	25.86
PHY0321	*Caldilineaceae*	12.44	1.61	27.47
PHY0204	*Cytophagaceae*	10.50	1.46	28.93
PHY0110	*Porphyromonadaceae*	10.35	1.38	30.31
PHY0847	*Rhodobacteraceae*	6.81	1.14	31.45
PHY1024	*Oxalobacteraceae*	7.57	1.06	32.50

B.

Average similarity among samples of the MD effluent: 39.95%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY0142	*Rikenellaceae*	49.29	8.04	8.04
PHY0321	*Caldilineaceae*	21.45	3.34	11.38
PHY1024	*Oxalobacteraceae*	12.43	2.02	13.40
PHY0291	*Anaerolinaceae*	10.17	1.83	15.23
PHY1009	*Comamonadaceae*	10.09	1.80	17.04
PHY1284	*Brachyspiraceae*	8.43	1.44	18.47
PHY0848	*Rhodobacteraceae*	8.14	1.28	19.75
PHY0847	*Rhodobacteraceae*	8.69	1.23	20.98
PHY0309	*Anaerolinaceae*	7.62	1.16	22.14
PHY0316	*Anaerolinaceae*	6.58	1.12	23.26
PHY0648	*Halanaerobiaceae*	9.25	1.10	24.36
PHY0844	*Rhodobacteraceae*	6.28	1.01	25.37
PHY1193	*Chromatiaceae*	13.08	1.00	26.37

Table S8 (continues).

C.

Average similarity among samples of Phase I: 55.94%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY0142	*Rikenellaceae*	45.89	3.54	3.54
PHY0321	*Caldilineaceae*	23.82	2.42	5.95
PHY1193	*Chromatiaceae*	19.30	1.47	7.42
PHY1009	*Comamonadaceae*	14.68	1.35	8.77
PHY1274	*Xanthomonadaceae*	15.06	1.27	10.04
PHY1024	*Oxalobacteraceae*	14.02	1.22	11.26
PHY1259	*Xanthomonadaceae*	11.57	1.07	12.33
PHY0110	*Porphyromonadaceae*	12.92	1.07	13.40
PHY0847	*Rhodobacteraceae*	9.92	0.94	14.34

D.

Average similarity among samples of Phase II: 25.73%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY0142	*Rikenellaceae*	25.51	12.13	12.13
PHY0384	*Turicibacteraceae*	30.83	10.34	22.47
PHY0245	Unclassified Candidate phylum Cloacimonetes (WWE1)	12.95	5.47	27.94
PHY0527	*Peptostreptococcaceae*	16.16	3.46	31.4
PHY0453	*Clostridiaceae*	15.42	3.43	34.83
PHY0407	*Clostridiaceae*	11.07	3.42	38.25
PHY0648	*Halanaerobiaceae*	10.77	2.96	41.2
PHY1009	*Comamonadaceae*	6.79	2.43	43.64
PHY0427	*Clostridiaceae*	10.01	2.16	45.80
PHY1021	*Oxalobacteraceae*	6.71	1.82	47.62
PHY0321	*Caldilineaceae*	10.07	1.72	49.34
PHY1024	*Oxalobacteraceae*	5.97	1.65	50.99

E.

Average dissimilarity among samples of the AcD and MD effluents: 69.08%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance, AcD**	**% Average relative abundance, MD**	**% Contribution**
PHY0384	*Turicibacteraceae*	23.98	10.53	1.79
PHY0142	*Rikenellaceae*	22.11	49.29	1.61
PHY0527	*Peptostreptococcaceae*	15.73	3.93	1.16
PHY0453	*Clostridiaceae*	14.14	3.90	1.03
PHY0321	*Caldilineaceae*	12.44	21.45	0.98
			% Cumulative contribution	6.57

Table S8 (continues).

F.

Average dissimilarity among samples of Phase I and II: 75.05%				
Phylotypes	**Taxonomic affiliation**	**% Average relative abundance, Phase I**	**% Average relative abundance, Phase II**	**% Contribution**
PHY0384	*Turicibacteraceae*	3.69	30.83	1.80
PHY0142	*Rikenellaceae*	45.89	25.51	1.32
PHY1193	*Chromatiaceae*	19.30	1.87	0.98
PHY0321	*Caldilineaceae*	23.82	10.07	0.94
PHY0527	*Peptostreptococcaceae*	3.50	16.16	0.88
PHY0453	*Clostridiaceae*	2.62	15.42	0.85
			% Cumulative contribution	6.76

Table S9. Pearson product-moment correlation coefficients (r) between the vectors in Figure 2A, 2B and 2C, which represent the relative abundances of the major bacterial Phyla (**A**) and Families (**B**) (average relative abundances >1%) detected by Illumina sequencing in the two stage-MAD samples in phases I and II, the operational parameters volatile solids/total solids percentage (%VS/TS), volatile fatty acids/alkalinity ratio (VFA/ALK), sludge retention time (SRT), organic loading rate (OLR), and the indicators of AD efficiency % of volatile solids removal (VSR), %CH4 in the biogas, and %CH4 recovery rate. Correlations **≥ 0.50** or **≤ -0.50** are boldfaced.

A)

Phyla	**%VS/TS**	**VFA/ALK**	**SRT**	**OLR**	**VSR**	**% CH_4 biogas**	**CH_4 recovery rate**
Actinobacteria	**-0.69**	**-1.00**	**0.98**	**-0.99**	**1.00**	**0.99**	**1.00**
Bacteroidetes	**-0.90**	-0.26	0.13	-0.18	0.31	0.45	0.36
***Ca.* Atribacteria**	**-0.83**	**-0.96**	**0.92**	**-0.94**	**0.98**	**1.00**	**0.99**
***Ca.* Cloacimonetes**	0.30	**-0.53**	**0.64**	**-0.60**	0.48	0.34	0.44
***Ca.* Saccharibacteria**	**0.67**	-0.13	0.26	-0.21	0.07	-0.08	0.02
Chloroflexi	**-0.81**	-0.09	-0.05	-0.01	0.14	0.29	0.19
Firmicutes	**0.67**	-0.13	0.26	-0.20	0.07	-0.08	0.02
Planctomycetes	**-0.96**	-0.42	0.30	-0.35	0.47	**0.60**	**0.51**
Proteobacteria	-0.45	0.39	**-0.51**	0.46	-0.34	-0.19	-0.29
Spirochaetes	**-0.69**	**-1.00**	**0.98**	**-0.99**	**1.00**	**0.99**	**1.00**
Synergistetes	**-0.66**	0.15	-0.27	0.22	-0.09	0.06	-0.04
Thermotogae	**-0.81**	-0.08	-0.05	-0.00	0.14	0.28	0.19

Table S9 (continues).

B)

Family	%VS/TS	VFA/ALK	SRT	OLR	VSR	% CH_4 biogas	CH_4 recovery rate
Anaerolinaceae	**-0.99**	**-0.61**	**0.56**	**-0.58**	**0.51**	0.10	0.38
Bradyrhizobiaceae	**-0.58**	0.16	-0.23	0.20	-0.28	**-0.66**	-0.42
Caldilineaceae	**-0.62**	0.11	-0.18	0.15	-0.23	**-0.63**	-0.37
Christensenellaceae	**0.92**	**0.93**	**-0.90**	**0.91**	**-0.88**	**-0.59**	**-0.80**
Chromatiaceae	-0.42	0.34	-0.41	0.38	-0.45	**-0.79**	-0.58
Clostridiaceae	**0.64**	-0.08	0.15	-0.12	0.20	**0.60**	0.34
Comamonadaceae	**-0.51**	0.25	-0.31	0.29	-0.36	**-0.73**	-0.49
Cytophagaceae	-0.24	**0.52**	**-0.57**	**0.55**	**-0.61**	**-0.89**	**-0.72**
Dethiosulfovibrionaceae	**-1.00**	**-0.77**	**0.73**	**-0.75**	**0.70**	0.32	**0.58**
Halanaerobiaceae	**0.56**	-0.19	0.26	-0.23	0.31	**0.68**	0.44
Hyphomicrobiaceae	**-0.84**	-0.21	0.14	-0.17	0.09	-0.34	-0.06
Intrasporangiaceae	**-1.00**	**-0.70**	**0.65**	**-0.67**	**0.61**	0.21	0.48
Lachnospiraceae	0.12	**0.78**	**-0.82**	**0.81**	**-0.85**	**-0.99**	**-0.92**
Moraxellaceae	**0.50**	**0.96**	**-0.98**	**0.97**	**-0.99**	**-0.96**	**-1.00**
Peptostreptococcaceae	**0.88**	0.29	-0.23	0.26	-0.18	0.26	-0.03
Porphyromonadaceae	-0.10	**0.63**	**-0.68**	**0.66**	**-0.72**	**-0.95**	**-0.81**
Propionibacteriaceae	**1.00**	**0.65**	**-0.59**	**0.62**	**-0.55**	-0.14	-0.42
Rhodobacteraceae	**-1.00**	**-0.70**	**0.65**	**-0.67**	**0.61**	0.21	0.49
Rhodocyclaceae	**0.70**	**1.00**	**-1.00**	**1.00**	**-0.99**	**-0.85**	**-0.97**
Rikenellaceae	**-1.00**	**-0.71**	**0.66**	**-0.68**	**0.62**	0.22	**0.50**
Ruminococcaceae	**1.00**	**0.66**	**-0.61**	**0.63**	**-0.57**	-0.16	-0.44
Saprospiraceae	**0.96**	**0.87**	**-0.84**	**0.85**	**-0.81**	-0.48	**-0.72**
Spirochaetaceae	**-0.91**	-0.36	0.29	-0.32	0.25	-0.19	0.10
Syntrophaceae	**-0.98**	**-0.83**	**0.79**	**-0.81**	**0.76**	0.41	**0.66**
Thermotogaceae	**-0.76**	-0.09	0.02	-0.05	-0.03	-0.46	-0.18
Turicibacteraceae	0.49	-0.26	0.33	-0.30	0.37	**0.74**	**0.51**
Xanthomonadaceae	0.04	**0.73**	**-0.78**	**0.76**	**-0.81**	**-0.98**	**-0.88**

Table S10. Phylotypes (PHYs) which mostly contributed to the global similarity of the archaeal communities between (A) samples of the acidogenic digester (AcD) effluent, (B) samples of the methanogenic digester (MD) effluent, (C) samples of both digesters in phase I (C), and (D) samples of both digesters in phase II (D), according to SIMPER analysis. (E) PHYs which mostly contribute to the dissimilarity of the archaeal communities between samples of the AcD and MD effluents (F) PHYs which mostly contributed to the dissimilarity of the archaeal communities of both digesters between samples of phases I and II, according to SIMPER analysis.

A.

Average similarity among samples of the AcD effluent: 51.55%				
Phylotype ID#	**Taxonomic affiliation**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY080	*Methanosarcina*	54.82	20.60	20.60
PHY048	*Methanolinea*	41.36	15.15	35.75
PHY045	*Methanolinea*	29.99	8.88	44.63
PHY015	*Methanobacterium*	12.86	6.54	51.17

B.

Average similarity among samples of the MD effluent: 43.55%				
Phylotype ID#	**Taxonomic affiliation**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY061	*Methanospirillum*	65.06	19.63	19.63
PHY048	*Methanolinea*	44.14	6.17	25.81
PHY052	*Methanospirillum*	32.15	5.97	31.78
PHY071	*Methanosaeta*	31.33	5.93	37.71
PHY058	*Methanospirillum*	16.33	5.57	43.29
PHY003	*Methanobacterium*	22.02	5.09	48.37
PHY080	*Methanosarcina*	26.81	4.89	53.26

C.

Average similarity among samples of Phase I: 56.59%				
Phylotype ID#	**Taxonomic affiliation**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY048	*Methanolinea*	73.45	21.88	21.88
PHY045	*Methanolinea*	55.46	15.14	37.02
PHY051	unclassified *Methanoregulaceae*	29.62	5.72	42.74
PHY080	*Methanosarcina*	19.71	5.16	47.90
PHY012	*Methanobacterium*	16.40	4.62	52.53

D.

Average similarity among samples of Phase II: 40.45%				
Phylotype ID#	**Taxonomic affiliation**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY080	*Methanosarcina*	61.92	29.72	29.72
PHY061	*Methanospirillum*	37.01	9.89	39.61
PHY015	*Methanobacterium*	12.99	4.99	44.59
PHY081	*Methanosarcina*	8.80	4.33	48.92
PHY048	*Methanolinea*	12.06	4.23	53.15

Table S10 (continues).

E.

Average dissimilarity among samples of the AcD and MD effluents: 68.56%				
Phylotype ID#	**Taxonomic affiliation**	**% Average relative abundance, AcD**	**% Average relative abundance, MD**	**% Contribution**
PHY061	*Methanospirillum*	3.29	65.06	13.63
PHY080	*Methanosarcina*	54.82	26.81	9.42
PHY048	*Methanolinea*	41.36	44.14	8.08
PHY045	*Methanolinea*	29.99	28.70	6.64
PHY052	*Methanospirillum*	5.78	32.15	4.78
PHY071	*Methanosaeta*	5.95	31.33	4.57
PHY051	unclassified *Methanoregulaceae*	8.83	21.58	3.45
			% Cumulative contribution	50.58

F.

Average dissimilarity among samples of Phase I and II: 69.41%				
Phylotype ID#	**Taxonomic affiliation**	**% Average relative abundance, Phase I**	**% Average relative abundance, Phase II**	**% Contribution**
PHY048	*Methanolinea*	73.45	12.06	11.88
PHY080	*Methanosarcina*	19.71	61.92	10.51
PHY045	*Methanolinea*	55.46	3.22	10.14
PHY061	*Methanospirillum*	31.34	37.01	8.98
PHY051	unclassified *Methanoregulaceae*	29.62	0.79	4.86
PHY071	*Methanosaeta*	28.72	8.57	3.99
			% Cumulative contribution	50.36

Table S11. Pearson product-moment correlation coefficients (r) between the vectors in Figure 2D and 2E, which represent the relative abundances of the 8 major methanogenic genera (average relative abundances >1%) detected by Illumina sequencing in the two stage-MAD samples in phases I and II, the operational parameters volatile solids/total solids percentage (%VS/TS), volatile fatty acids/alkalinity ratio (VFA/ALK), sludge retention time (SRT), organic loading rate (OLR), and the indicators of AD efficiency % of volatile solids removal (VSR), %CH4 in the biogas, and %CH4 recovery rate. Correlations **≥ 0.50** or **≤ -0.50** are boldfaced.

Genera	%VS/TS	VFA/ALK	SRT	OLR	VSR	% CH_4 biogas	CH_4 recovery rate
Methanobacterium	**-0.68**	0.25	-0.21	0.26	-0.27	-0.41	-0.48
Methanobrevibacter	-0.17	**0.74**	**-0.71**	**0.75**	**-0.76**	**-0.85**	**-0.88**
Methanocorpusculum	**0.55**	**1.00**	**-1.00**	**1.00**	**-1.00**	**-0.98**	**-0.97**
Methanoculleus	**-0.65**	**-0.99**	**1.00**	**-0.99**	**0.99**	**0.95**	**0.93**
Methanolinea	-0.02	**0.83**	**-0.81**	**0.84**	**-0.84**	**-0.92**	**-0.94**
Methanosaeta	**-0.99**	**-0.66**	**0.69**	**-0.65**	**0.64**	**0.52**	0.46
Methanosarcina	**0.93**	0.20	-0.24	0.18	-0.17	-0.02	0.05
Methanospirillum	**-0.81**	**-0.93**	**0.94**	**-0.92**	**0.92**	**0.85**	**0.81**

Table S12. Phylotypes (PHYs) which mostly contributed to the global similarity of the fungal communities between (A) samples of the acidogenic digester (AcD) effluent, and (B) samples of the methanogenic digester (MD) effluent, in phase I, according to SIMPER analysis. (C) PHYs which mostly contributed to the dissimilarity of the fungal communities between samples of the AcD and MD effluents in phase I, according to SIMPER analysis.

A.

Average similarity among samples of the AcD effluent: 42.54%				
Phylotypes	**Taxonomic affiliation (Order level)**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY176	*Saccharomycetales*	15.07	14.42	14.42
PHY182	*Saccharomycetales*	12.94	8.02	22.44
PHY158	*Saccharomycetales*	9.17	5.04	27.48
PHY105	*Hypocreales*	6.29	4.79	32.27
PHY148	*Saccharomycetales*	4.79	4.25	36.52
PHY052	*Eurotiales*	7.69	3.95	40.47
PHY043	*Eurotiales*	6.57	3.81	44.28
PHY157	*Saccharomycetales*	3.81	3.73	48.01
PHY057	*Eurotiales*	3.29	2.79	50.79

B.

Average similarity among samples of the MD effluent: 45.37%				
Phylotypes	**Taxonomic affiliation (Order level)**	**% Average relative abundance**	**% Contribution**	**% Cumulative contribution**
PHY052	*Eurotiales*	16.74	7.87	7.87
PHY176	*Saccharomycetales*	15.84	7.04	14.91
PHY182	*Saccharomycetales*	14.92	6.83	21.74
PHY043	*Eurotiales*	14.01	6.71	28.46
PHY113	*Microascales*	10.94	5.42	33.88
PHY105	*Hypocreales*	10.07	4.01	37.89
PHY057	*Eurotiales*	7.60	3.53	41.41
PHY222	Unclassified *Microbotryomycetes*	7.23	3.04	44.45
PHY165	*Saccharomycetales*	9.08	2.89	47.34
PHY148	*Saccharomycetales*	7.72	2.53	49.87
PHY232	*Mortierellales*	7.42	2.37	52.24

Table S12 (continues)

C.

Average dissimilarity among samples of the AcD and MD effluents: 61.89%				
Phylotypes	**Taxonomic affiliation (Order level)**	**% Average relative abundance, AcD**	**% Average relative abundance, MD**	**% Contribution**
PHY052	*Eurotiales*	7.69	16.74	2.74
PHY043	*Eurotiales*	6.57	14.01	2.18
PHY182	*Saccharomycetales*	12.94	14.92	2.14
PHY165	*Saccharomycetales*	3.62	9.08	2.03
PHY113	*Microascales*	3.89	10.94	1.91
PHY232	*Mortierellales*	3.12	7.42	1.73
PHY158	*Saccharomycetales*	9.17	4.17	1.71
PHY073	Unclassified *Pezizomycotina*	0.00	5.97	1.48
PHY222	Unclassified *Pucciniomycotina*	2.19	7.23	1.44
PHY211	*Trichosporonales*	1.63	6.02	1.43
PHY098	*Hypocreales*	4.47	5.43	1.39
PHY105	*Hypocreales*	6.29	10.07	1.38
PHY082	*Pezizales*	0.00	5.15	1.37
PHY185	*Saccharomycetales*	0.86	5.66	1.33
PHY148	*Saccharomycetales*	4.79	7.72	1.33
			% Cumulative contribution	25.58

Table S13. Pearson product-moment correlation coefficients (r) between the vectors in Figure 4, which represent the relative abundances of the major bacterial Phyla (**A**) and Families (**B**) and methanogenic archaea genera (**C**) (average relative abundances >1%) detected by Illumina sequencing in the two stage-MAD samples in phases I and II, and the REs of the PhACs atenolol, fenofibrate, ketoprofen, naproxen, ofloxacin and trimethoprim. Correlations **≥ 0.50** or **≤ -0.50** are boldfaced.

A.

Phyla	Atenolol	Fenofibrate	Ketoprofen	Naproxen	Ofloxacin	Trimethoprim
Actinobacteria	0.04	**-0.83**	**0.97**	**0.93**	**-0.76**	**0.59**
Bacteroidetes	**-0.50**	0.47	**-0.75**	**-0.66**	**0.98**	-0.15
***Ca.* Atribacteria**	**0.99**	0.42	-0.07	-0.20	**-0.77**	**-0.70**
***Ca.* Cloacimonetes**	**0.77**	-0.13	0.47	0.35	**-0.99**	-0.21
***Ca.* Saccharibacteria**	**0.65**	-0.30	**0.62**	**0.51**	**-1.00**	-0.04
Chloroflexi	0.46	**1.00**	**-0.96**	**-0.99**	0.34	**-0.91**
Firmicutes	0.09	**-0.80**	**0.96**	**0.91**	**-0.79**	**0.55**
Planctomycetes	**0.97**	0.32	0.03	-0.10	**-0.83**	**-0.62**
Proteobacteria	-0.12	**0.78**	**-0.95**	**-0.90**	**0.81**	**-0.53**
Spirochaetes	**0.78**	**0.94**	**-0.76**	**-0.84**	-0.08	**-1.00**
Synergistetes	**-0.92**	-0.16	-0.20	-0.07	**0.91**	0.48
Thermotogae	0.27	**0.96**	**-1.00**	-0.10	**0.52**	**-0.81**

B.

Family	Atenolol	Fenofibrate	Ketoprofen	Naproxen	Ofloxacin	Trimethoprim
Anaerolinaceae	**0.51**	**1.00**	**-0.94**	**-0.98**	0.28	**-0.94**
Bradyrhizobiaceae	-0.25	**0.69**	**-0.90**	**-0.84**	**0.88**	-0.41
Caldilineaceae	0.20	**0.94**	**-1.00**	**-0.99**	**0.58**	**-0.77**
Christensenellaceae	**-0.52**	**-1.00**	**0.94**	**0.98**	-0.27	**0.94**
Chromatiaceae	**-0.65**	0.30	**-0.62**	**-0.51**	**1.00**	0.04
Clostridiaceae	0.13	**-0.77**	**0.95**	**0.90**	**-0.81**	**0.52**
Comamonadaceae	0.16	**0.92**	**-1.00**	**-0.99**	**0.62**	**-0.74**
Cytophagaceae	**-0.51**	**-1.00**	**0.94**	**0.98**	-0.28	**0.94**
Dethiosulfovibrionaceae	**0.61**	**1.00**	**-0.90**	**-0.95**	0.17	**-0.97**
Halanaerobiaceae	-0.18	**-0.93**	**1.00**	**0.99**	**-0.59**	**0.76**
Hyphomicrobiaceae	0.45	**1.00**	**-0.96**	**-0.99**	0.34	**-0.91**
Intrasporangiaceae	**0.99**	0.38	-0.03	-0.16	**-0.79**	**-0.67**
Lachnospiraceae	**-0.53**	0.44	**-0.73**	**-0.63**	**0.98**	-0.12
Moraxellaceae	**-0.50**	0.47	**-0.75**	**-0.66**	**0.98**	-0.15
Oxalobacteraceae	**0.87**	**0.88**	**-0.65**	**-0.75**	-0.23	**-0.99**
Peptostreptococcaceae	-0.25	**-0.95**	**1.00**	**1.00**	**-0.54**	**0.80**
Porphyromonadaceae	-0.23	**0.70**	**-0.91**	**-0.85**	**0.87**	-0.43
Propionibacteriaceae	-0.08	**-0.89**	**0.99**	**0.97**	**-0.67**	**0.69**
Rhodobacteraceae	0.35	**0.98**	**-0.99**	**-1.00**	0.45	**-0.86**
Rhodocyclaceae	**-0.60**	0.36	**-0.67**	**-0.56**	**0.99**	-0.03
Rikenellaceae	-0.45	**0.52**	**-0.79**	**-0.70**	**0.96**	-0.21
Ruminococcaceae	**-0.69**	**-0.98**	**0.84**	**0.91**	-0.06	**0.99**
Saprospiraceae	**-0.98**	**-0.69**	0.38	**0.51**	**0.52**	**0.90**
Spirochaetaceae	**-0.99**	**-0.67**	0.36	0.48	**0.55**	**0.88**
Syntrophaceae	**0.86**	0.02	0.34	0.21	**-0.96**	-0.35
Thermotogaceae	0.27	**0.96**	**-1.00**	**-1.00**	**0.52**	**-0.81**
Turicibacteraceae	0.47	**-0.50**	**0.77**	**0.68**	**-0.97**	0.18
Xanthomonadaceae	**-0.87**	-0.05	-0.31	-0.18	**0.95**	0.37

Table S13 (continues)

C.

Genera	Atenolol	Fenofibrate	Ketoprofen	Naproxen	Ofloxacin	Trimethoprim
Methanobacterium	0.42	**0.99**	**-0.97**	**-0.99**	0.38	**-0.89**
Methanobrevibacter	**-0.66**	0.29	**-0.61**	**-0.50**	**1.00**	0.04
Methanocorpusculum	-0.06	**0.81**	**-0.97**	**-0.92**	**0.77**	**-0.58**
Methanoculleus	**0.74**	-0.18	**0.51**	0.39	**-1.00**	-0.16
Methanolinea	**-0.65**	0.30	**-0.62**	**-0.51**	**1.00**	0.03
Methanosaeta	**-0.59**	0.37	**-0.68**	**-0.57**	**0.99**	-0.05
Methanosarcina	-0.17	**-0.93**	**1.00**	**0.99**	**-0.60**	**0.75**
Methanospirillum	**0.84**	-0.02	0.38	0.25	**-0.97**	-0.31

Figure S1. Schematic diagram of the two-stage mesophilic anaerobic digestion pilot-scale plant use in this study. Green co
colour to the sludge line.

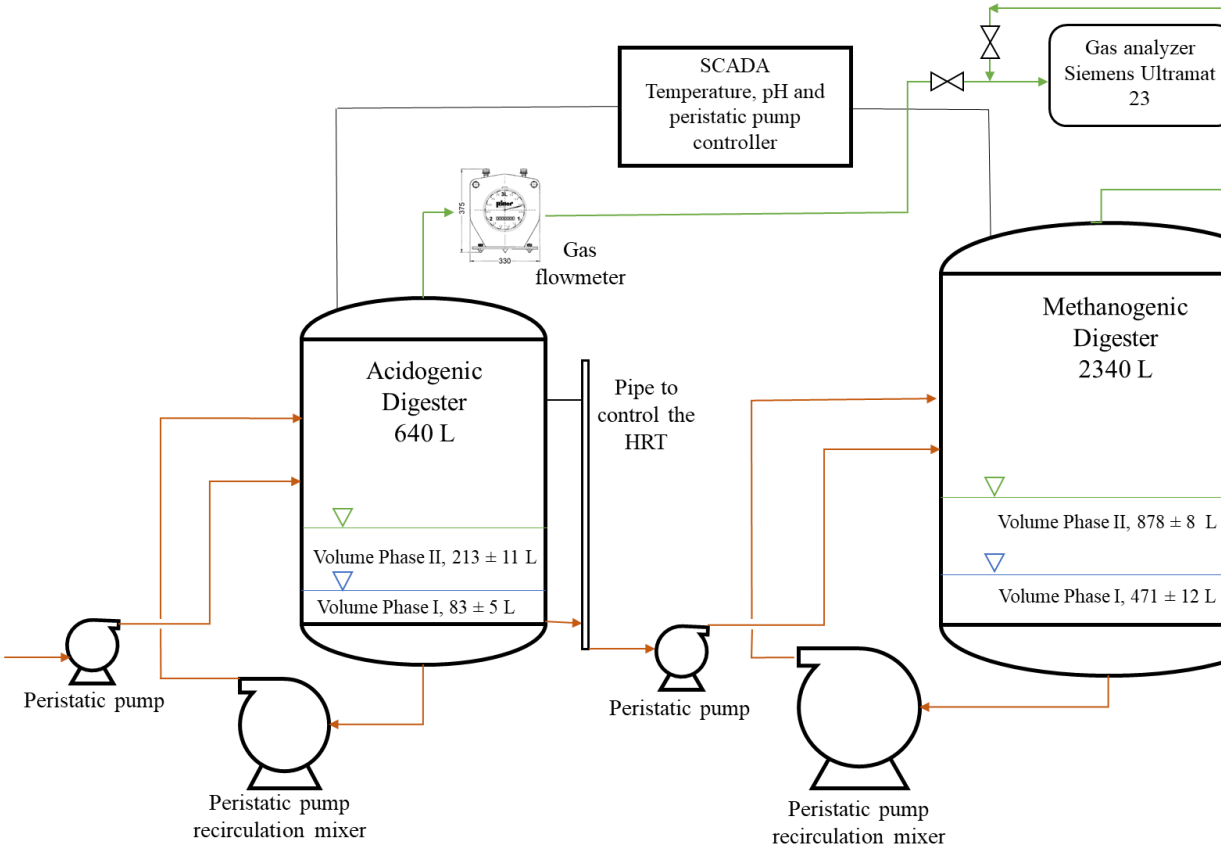


Figure S2. Schematic diagram of the wastewater pilot-scale plant use in this study. Sampling points 1, 2 and 3 correspond to sludge effluent (AcS) and methanogenic sludge effluent (MS) respectively. Blue colour corresponds to the water line and b

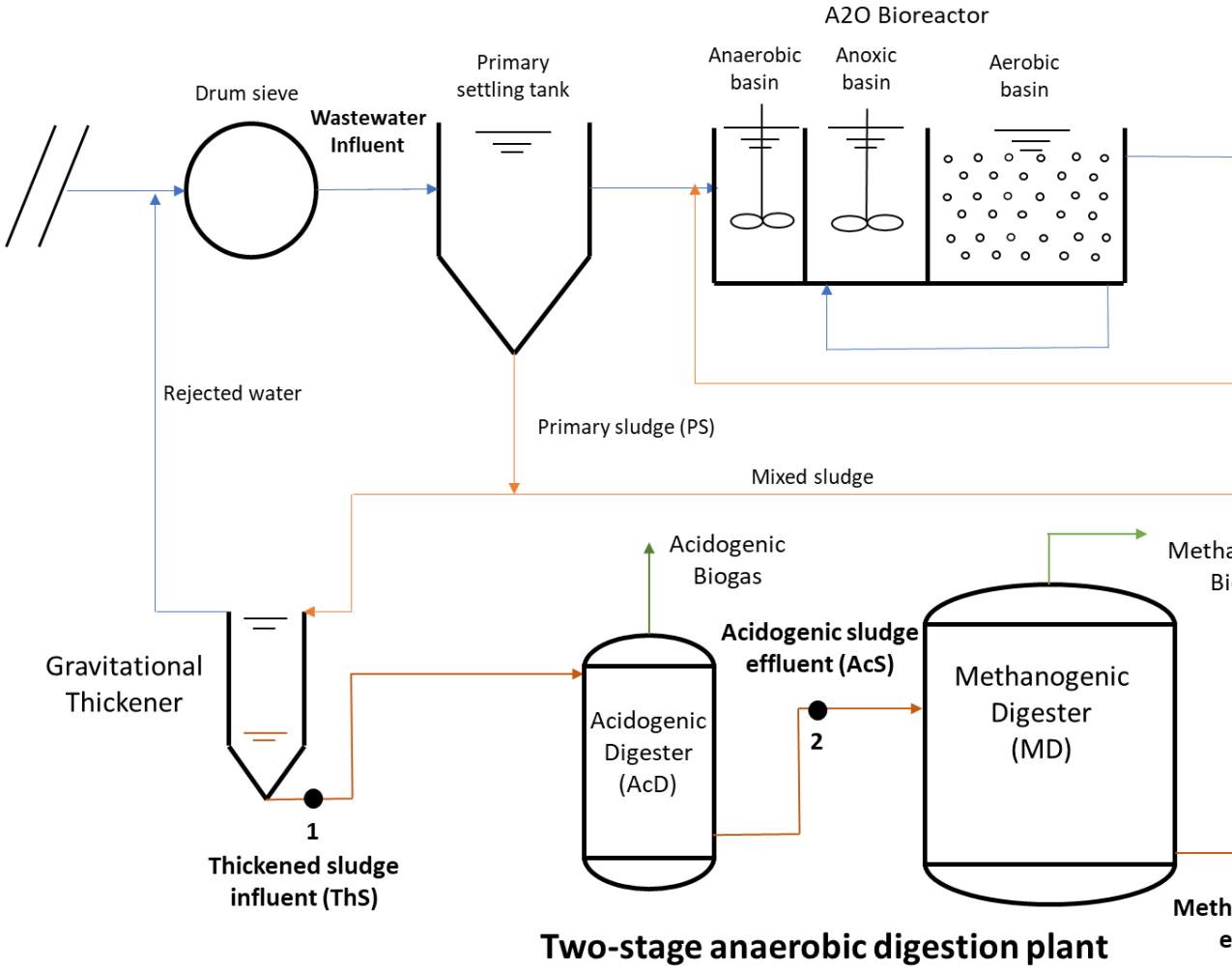


Figure S3. Quality Control (QC) - Extracted Ion Chromatograms (XIC) of target compounds in in positive electrospray ionization by UPLC-HR-QTOF-MS.

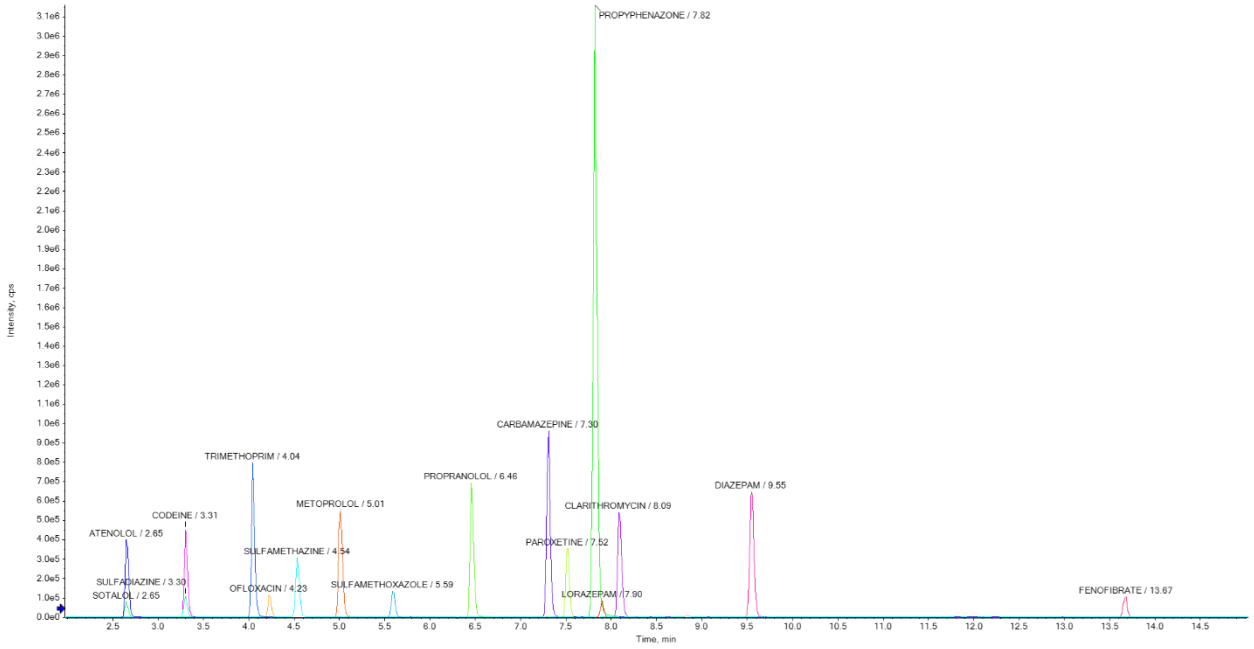


Figure S4. Quality Control (QC) - Extracted Ion Chromatograms (XIC) of surrogate compounds in positive electrospray ionization by UPLC-HR-QTOF-MS.

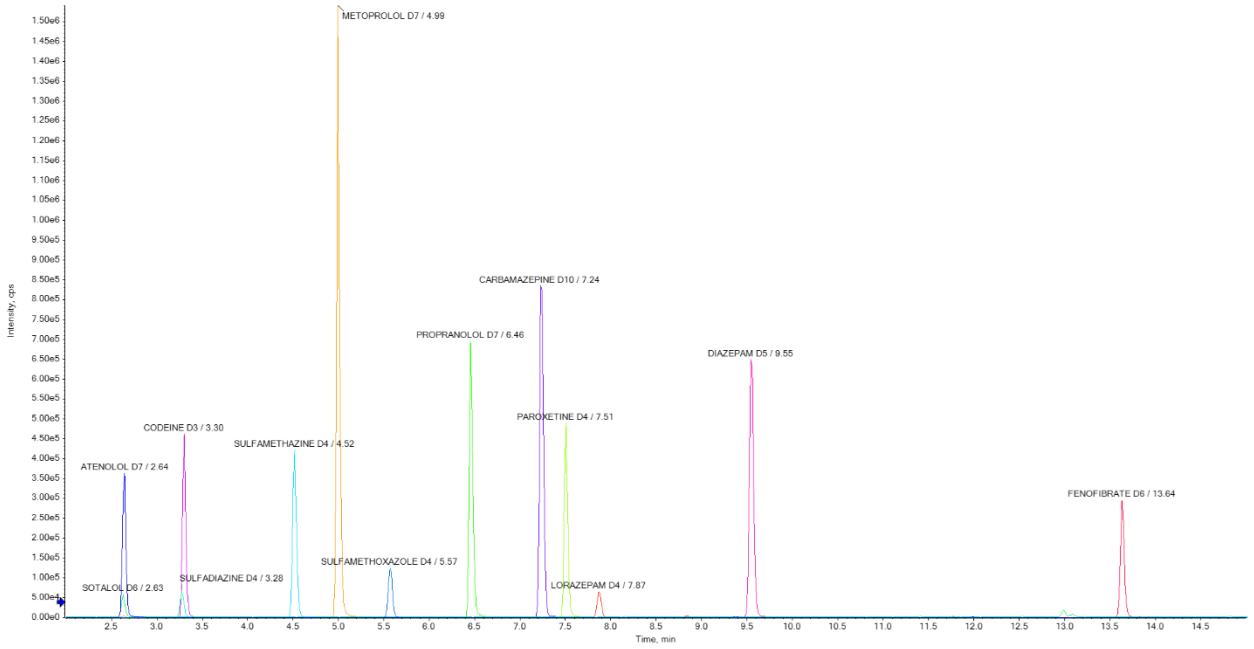


Figure S5. Quality Control (QC) - Extracted Ion Chromatograms (XIC) of target compounds in in negative electrospray ionization by UPLC-HR-QTOF-MS.

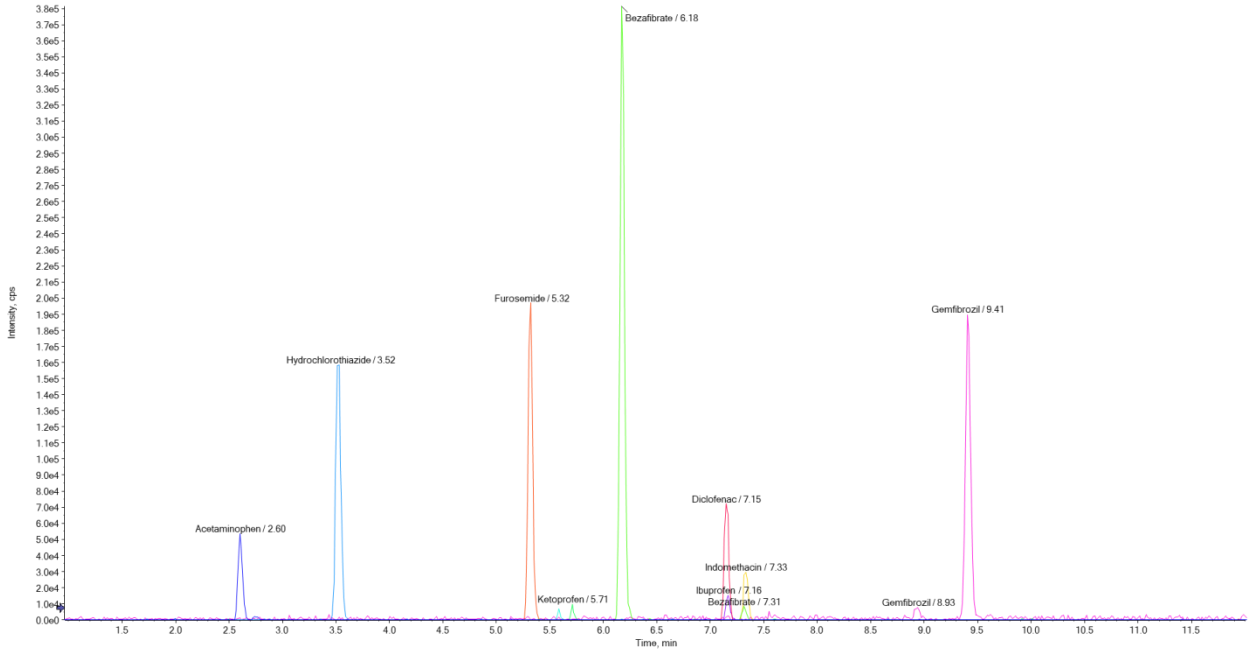


Figure S6: Quality Control (QC) - Extracted Ion Chromatograms (XIC) of surrogate compounds in negative electrospray ionization by UPLC-HR-QTOF-MS.

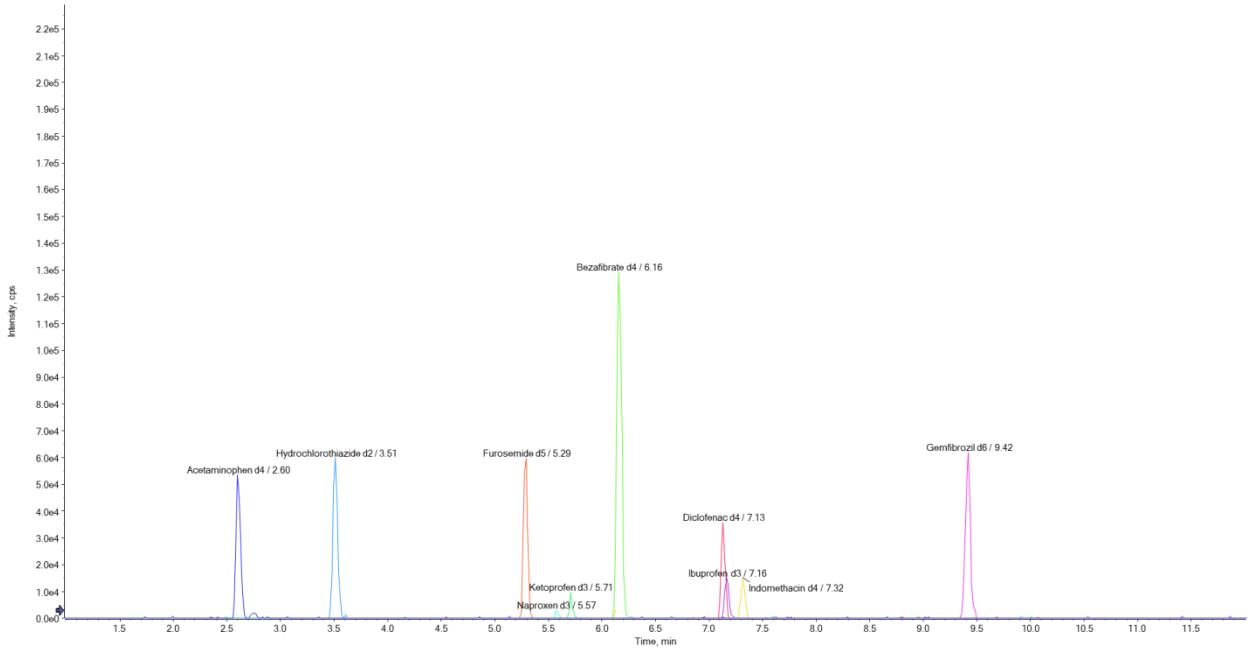


Figure S7. Sample-size-based and coverage-based rarefaction and extrapolation (R/E) phylotype accumulation curves (wi
Bacteria, Archaea and Fungi in the acidogenic digester (AcD) and methanogenic digester (MD) effluent samples, through
lines are estimates of richness via interpolation, while dashed lines are extrapolations of richness for a fair comparison of u

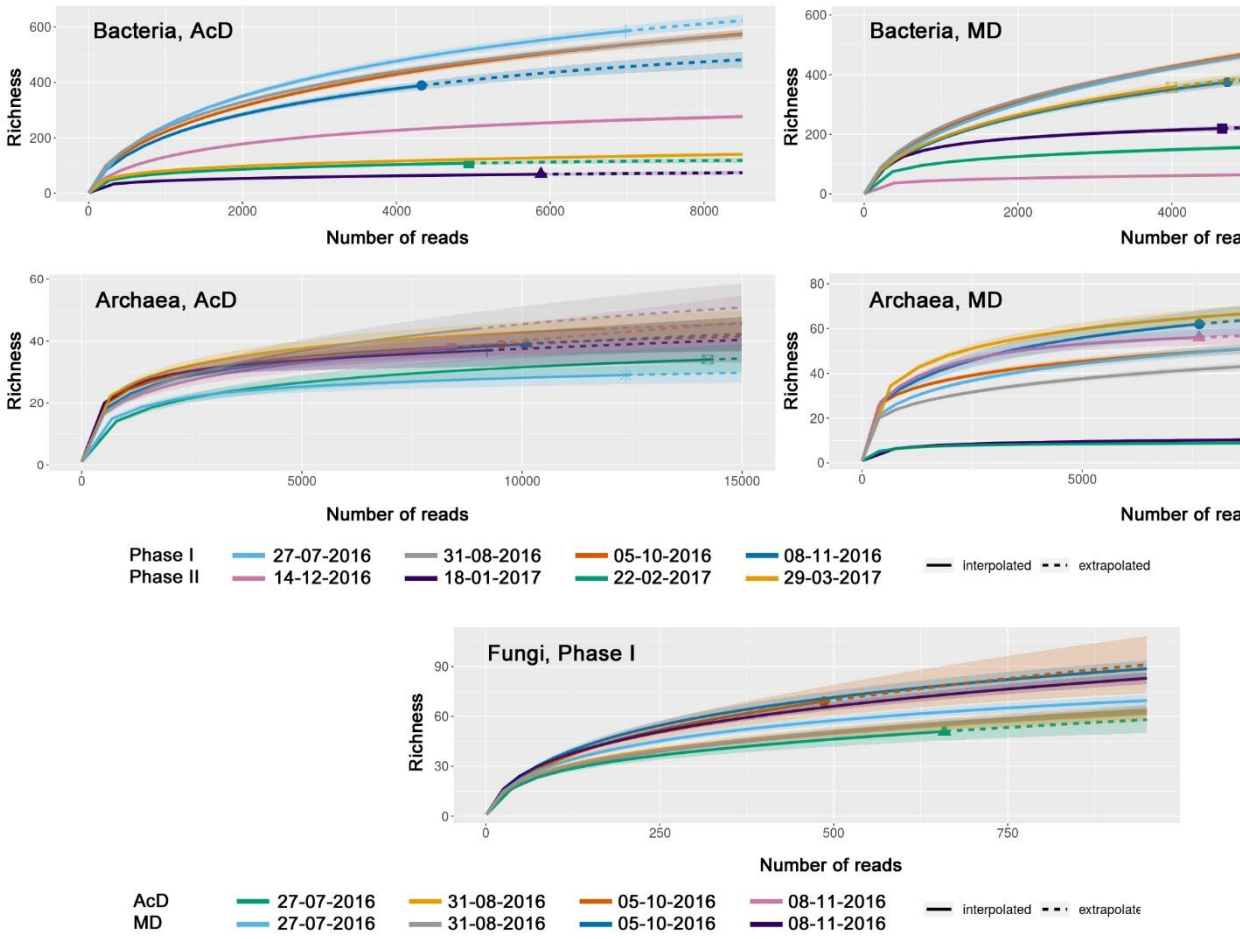


Figure S8. Dendrograms showing the similarities among the Bacteria (A), Archaea (B) and Fungi (C) communities in the acidogenic digester (AcD) and methanogenic digester (MD) effluent samples throughout the experimental phases I and II, based on the analysis of the small-subunit rRNA sequences. Clustering was generated by the UPGMA algorithm using the Bray-Curtis distance similarity matrix generated from the abundance of reads of the phylotypes identified by Illumina-sequencing. Lines in red mark the non-significantly different branches, according to SIMPROF analysis ($p>0.05$).

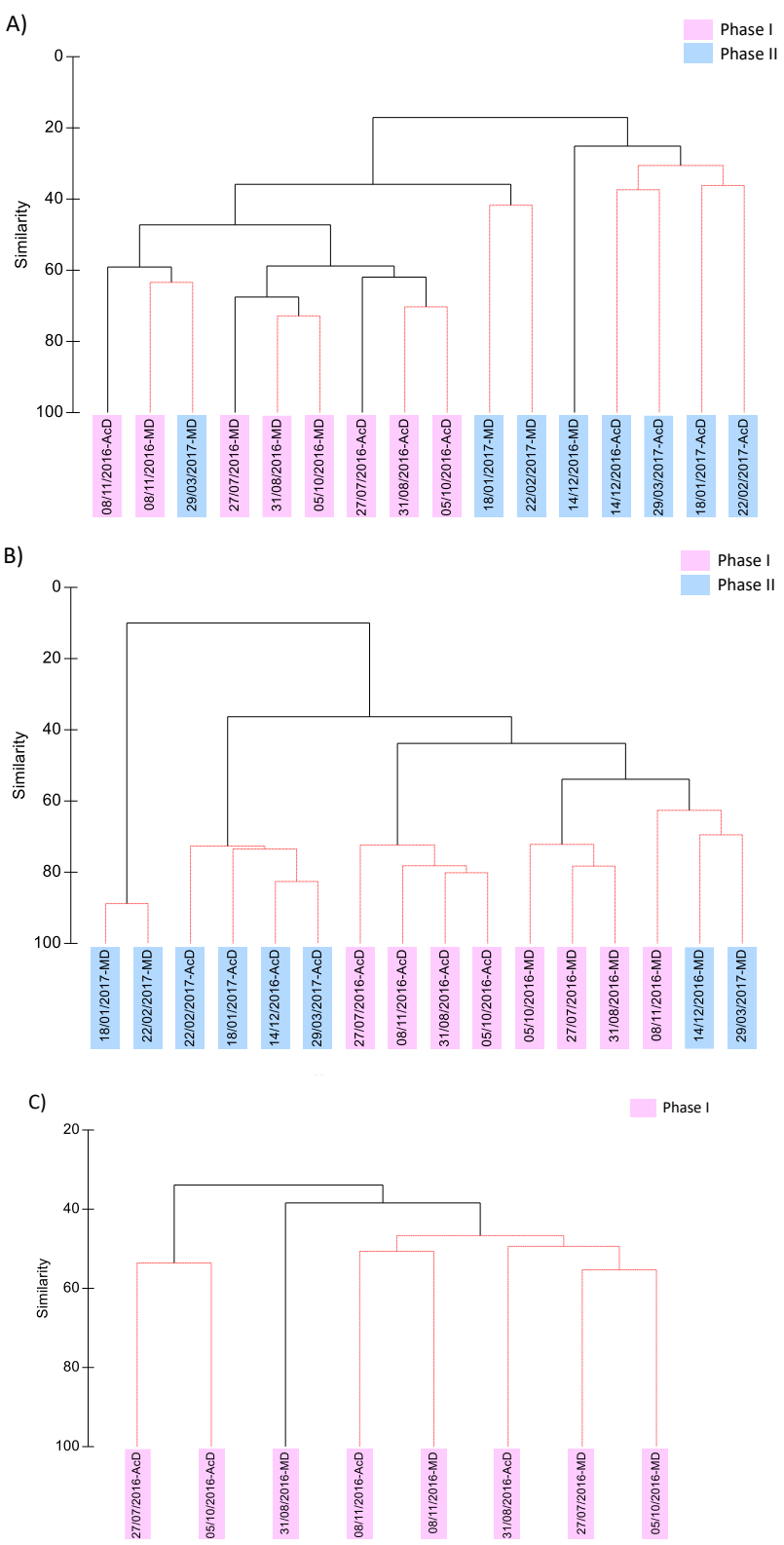


Figure S9. Heatmap displaying the relative abundances of Bacteria phylotypes (Family level), detected by Illumina sequencing in the acidogenic digestor (AcD) and the methanogenic digestor (MD) effluent samples in the experimental phases I and II. Only Families with ≥1% relative abundance in at least 1 sample were included.

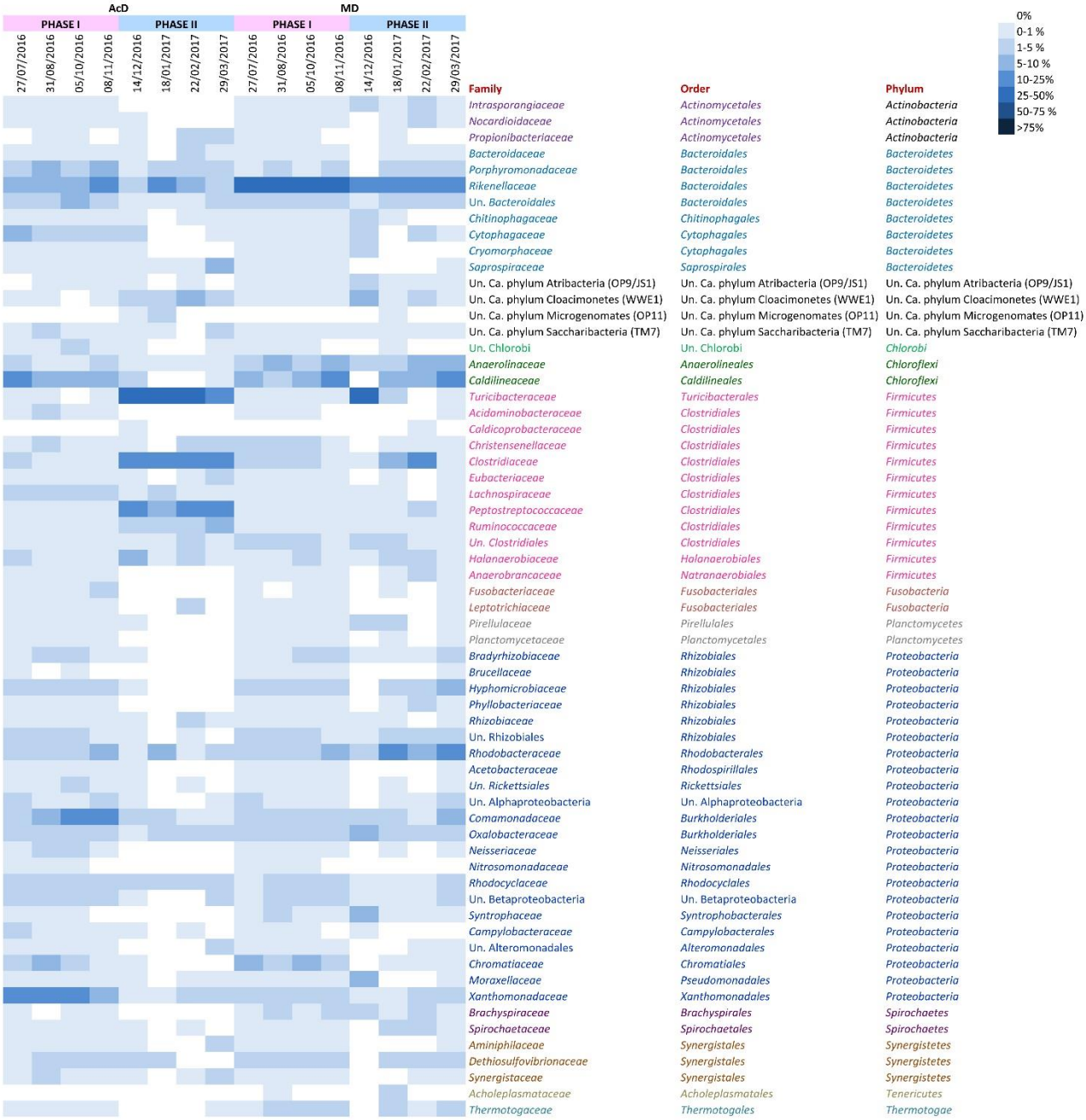
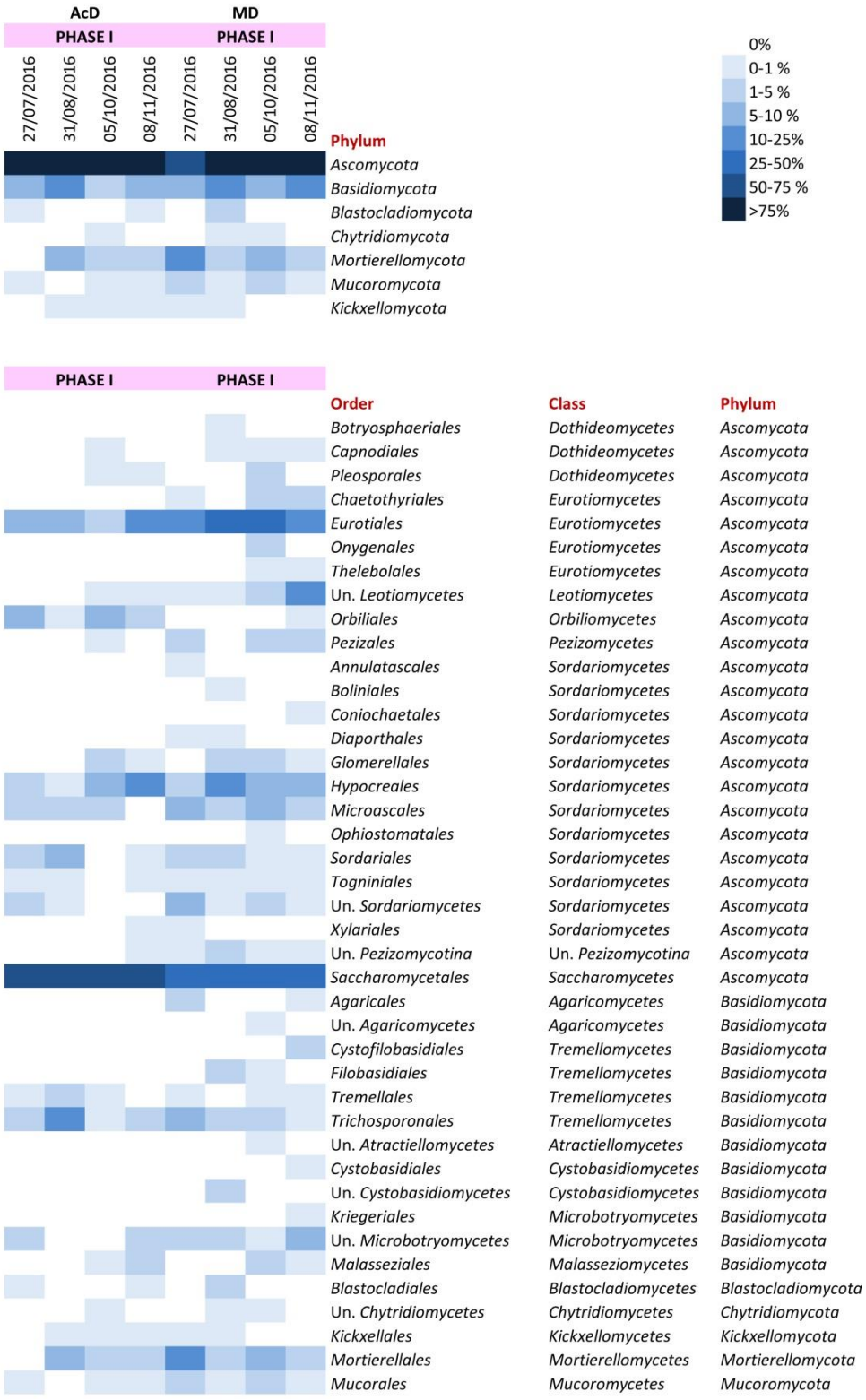


Figure S10. Heatmap displaying the relative abundances of Fungi phylotypes (Phylum and Order levels), detected by Illumina sequencing in the acidogenic digestor (AcD) and the methanogenic digestor (MD) in experimental phase I.



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❖ CHAPTER 4

Fate of pharmaceutically active compounds in a pilot-scale A^2O integrated fixed-film activated sludge (IFAS) process treating municipal wastewater

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Abstract

The goal of this research was to study the operation of an IFAS, Integrated Fixed-film Activated Sludge, system, in A^2O configuration, Anaerobic/Anoxic/Aerobic, for the elimination of emerging pollutants, in particular of 27 PhACs, Pharmaceutical Active Compounds (eight anti-inflammatory and/or analgesic pharmaceuticals (AIAPs), six antibiotics, four b-blockers, two antihypertensives/diuretics, three lipid regulators-bezafibrate and four psychiatric medications). Different operational conditions were analyzed in the biological reactor, controlling at all times both the amount of suspended and fixed biomass present. The A^2O -IFAS system has obtained similar or higher removal efficiency (RE) in elimination of organic matter, and especially of nutrients, N and P, with respect to the conventional A^2O system, working however with very low values of the sludge retention time (SRT) (4.0 days) and MLSS in the reactor (1822 mg L-1), thanks to the contribution of the fixed biomass. In general, CAS and IFAS processes show the importance of operate at high SRT and MLSS concentration, that give low F/M ratio, to increase the RE of several PhACs. However, the A2O-IFAS system, operating with low mixed liquor SRT and MLSS has achieved similar or better RE of PhACs, obtaining the highest average REs values (>80%) for fenofibrate, acetaminophen, ibuprofen, naproxen, clarithromycin and atenolol. According to the results obtained, due to the affordable cost

and well-demonstrated performance, IFAS systems become one of the most promising technologies for conventional WWTP upgrading.

1. Introduction

During the last decades, the production of nutrient-rich wastewaters has been continuously increasing worldwide due to the exponential growth of urbanization, wastewater flow, household consumption and industrial production (EEA, 2018). Accordingly, the continuous discharge of nutrients in water bodies leads to an increasing eutrophication problem. Moreover, the continues presence of different trace organic contaminants such as personal care products, industrial chemicals, hormones and more specific Pharmaceutical Active Compounds (PhACs) in municipal wastewaters effluents and different environmental compartments is an issue of growing concern worldwide (aus der Beek et al., 2016). Nowadays, the conventional wastewater treatment plants (WWTPs) have demonstrated many shortcomings to face more stringent discharge standards in terms of nutrient release and emerging contaminants removal (COM(2019) 128, 2019; Güneş et al., 2019). Consequently, the old WWTPs require upgrading (e.g. construction of new aeration tanks and secondary clarifiers) and the implementation of new advanced biological treatment processes. In the last decades, conventional biological nutrient removal (BNR) processes have been well characterized and obtain good performance. However, they need massive reactor volumes and operate at high sludge retention time (SRT), especially in cold weather, which often required high energy and investment costs (Ashrafi et al., 2019). Additionally, WWTPs were not designed to remove trace organic contaminants and only a fraction of each PhAC and their metabolites can be removed (Grandclément et al., 2017; Verlicchi et al., 2012).

To overcome these problematics, several investigations have been published regarding the fate and removal efficiencies (REs) of PhACs in WWTPs, as well as, to solve the main drawbacks of the conventional BNR configurations (Ashrafi et al., 2019; Grandclément et al., 2017; Verlicchi et al., 2012). Among the novel technologies, the moving bed biofilm reactors (MBBR) and more specifically the hybrid MBBR-based integrated fixed-film activated sludge (IFAS) reactors are shown as promising technologies due to their advantages compared to the conventional activated sludge (CAS) processes. During the last decade, IFAS system has gained acceptance as a cost-effective technology to enhance nitrogen removal, improve process stability, enhance settleability and increase

the overall treatment capacity of the conventional WWTPs (di Biase et al., 2019; Leyva-Díaz et al., 2020). Furthermore, recent investigations suggest better removal efficiencies of some PhACs by IFAS process compared to CAS and membrane bioreactor (MBR) systems (Ben et al., 2018; De La Torre et al., 2015; Falås et al., 2016; Jewell et al., 2016; Luo et al., 2015; Ooi et al., 2018; Shreve and Brennan, 2019). The main advantage of IFAS processes is the presence of both quick-growth suspended and slow-growth attached microorganisms in the same reactor compartment. Thus, application of attached microorganisms allows to have biomass with higher sludge retention time (SRT) in the biofilm for the nitrification process and lower SRT in the suspended biomass for the biodegradation of organic matter, which the possibility to operate at higher biomass concentration compared to conventional BNR processes (Ashrafi et al., 2019). Indeed, this process became a very simple and efficient technology for upgrading overloaded WWTPs or design a new municipal WWTP (di Biase et al., 2019; Leyva-Díaz et al., 2017).

Despite the aforementioned benefits of IFAS systems, there are scarce studies investigating, in the same pilot-scale plant with real wastewater, the removal efficiency of PhACs in IFAS systems compared to CAS and MBR systems (Shreve and Brennan, 2019). Falås et al. (2012, 2013) and Jewell et al. (2016) demonstrated during bench-scale batch experiments using both activated sludge and suspended biofilm carrier from full-scale WWTPs that attached biomass could contribute significantly to the removal of some PhACs. Similar results were obtained in several pilot-scale assessments in IFAS systems (De La Torre et al., 2015; Luo et al., 2015). These studies pointed out that biodegradation/biotransformation served as the primary pathway for PhACs removal and the importance of the operating condition such as SRT and biomass concentration (MLSS). However, more research is needed to fully explore the higher RE capacity and the underlying removal mechanisms in IFAS systems, since there is still a lack of information between the RE of PhACs and the impact of operating conditions on the ability of IFAS process to transform PhACs compered to CAS process.

In this study, the removal rate of organic matter and nutrients, as well as, the removal of twenty seven PhACs (eight anti-inflammatory and/or analgesic pharmaceuticals (AIAPs)- acetaminophen, ibuprofen, naproxen, ketoprofen, diclofenac, codeine, indomethacin and propyphenazone-, six antibiotics-clarithromycin, ofloxacin, sulfadiazine, sulfamethazine, sulfamethoxazole and trimethoprim-, four b-blockers -atenolol, metoprolol, propranolol and sotalol-, two antihypertensives/diuretics -furosemide and hydrochlorothiazide-, three lipid regulators-bezafibrate, fenofibrate and gemfibrozil-,

and four psychiatric medications -carbamazepine, diazepam, lorazepam and paroxetine) was evaluated in a pilot-scale anaerobic/anoxic/aerobic (A^2O) plant operating as IFAS process with 50% of the aerobic basin filled with the carrier AnoxKaldnes K5. The A^2O-IFAS pilot-scale plant was long-term operated treating real wastewater coming from the pretreatment unit of the full-scale WWTP Murcia Este. The linking between the removal rates of the targeted PhACs, the changes in environmental/operating variables, and the removal rates of organic matter and nutrients was evaluated by means of Spearman's rank correlations coefficients.

2. Materials and methods

2.1. Description of the wastewater treatment pilot-scale plant

The pilot-scale A^2O-IFAS plant (Figure 1) was located at the facilities of the WWTP *Murcia Este* (EMUASA, Murcia, Spain). The characteristic of the pilot-scale plant has been previously described in full detail by Gallardo-Altamirano et al. (2018). In brief, the pilot-scale plant is designed to treat up to 6 m^3 per day of wastewater coming from the pretreatment unit of the full-scale WWTP Murcia Este. The pilot plant was previously operated and studied as conventional BNR A^2O system during two experimental phases (from May 20th, 2016 to March 31st, 2017) with different operational/environmental conditions (Gallardo-Altamirano et al., 2019, 2018). After the second phase, the pilot-scale A^2O plant was converted to IFAS system by filling the aerobic basin (1.20 m^3) at 50% with AnoxKaldnes K5 carrier, which correspond to 0.36 filling ratio of the total bioreactor volume. Table 1 shown the main characteristics of the carrier used with a carrier photograph with and without attached biomass. The start-up and the stabilization phase of IFAS system lasted 92 days (from 2nd May to 2nd August 2017). Since 1st July 2017 the stabilization phase started to operate with the selected working concentration of mixed liquor suspended solids (MLSS) between 1500–2000 mg L^{-1}, biofilm suspended solid (BFSS) between 1000-2000 mg L^{-1}, mixed liquor sludge retention time (ML-SRT) between 3.5-4.5 days, and the mixed liquor food-to-microorganism ratio (ML-F/M) over 0.40 $kgBOD_5$ $kgMLVSS^{-1}$ d^{-1}. The experimental phase started up on 2nd August 2017 after the steady-state was reached (more than threefold SRT) and lasted 105 days. Monthly average values ± standard deviations for operational parameters, and performance rates of the A^2O-IFAS system are shown in Table 2 and the average values ± standard for physico/chemical

concentration measured in the decanted influent and effluent (point 2 and 3 respectively, Figure 1) are shown in the Table 3.

Figure 1. Schematic diagram of the pilot-scale A^{2}O-IFAS plant used in the study. Sampling points 1, 2 (influent) ar

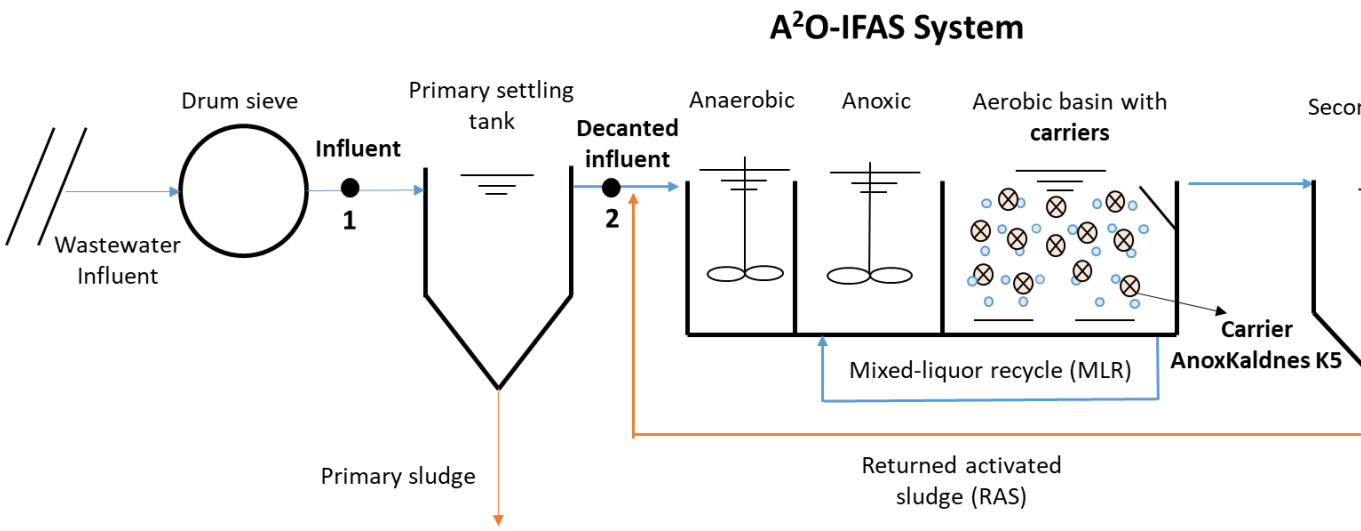
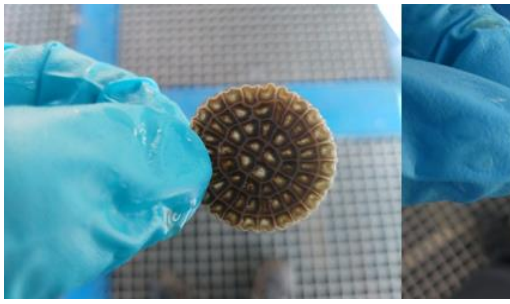


Table 1. Main characteristics of AnoxKaldnes K5 carrier

Characteristics	AnoxKaldnes K5	Carrier picture
Material	High-density polyethylene	
Shape	Cylinder	
Density, kg L^{-1}	0.95	
Bulk Density, Kg m^{-3}	118	
Specific surface area, m^2 m^{-3}	800	
Nominal diameter, mm	25	
Nominal thickness, mm	3.5	
Count per m^3	331000	



2.2. Wastewater sampling collection for the analysis of physicochemical parameters and PhACs

To determine the physicochemical parameters shown in the Table 2 and 3, daily-composite samples were taken three times per week from the sampling points 2 and 3 (Figure 1). Chemical oxygen demand (COD), total nitrogen (TN), $N\text{-}NO_3^-$,$N\text{-}NH_4^+$ and total phosphorous (TP) were measured by Merck Spectroquant® kits (Darmstadt, Germany), while MLSS, volatile suspended solids (MLVSS), total suspended solids (TSS), biological oxygen demand (BOD_5), were measured according to standard methods (SM 2540 for MLSS, MLVSS and TSS; SM 5210B for BOD_5) (Baird et al., 2017). The biomass concentration (BFSS) attached in the plastic carriers was achieved as follows: twelve representative carriers were removed from the bioreactor, diluted in 50 ml of distillated water with Tween 80 (1/1000 of dilution), sonicated for 15 minutes and centrifugated for 20 min at 3000 rpm. Once the biomass was separated from the plastic carriers, the BFSS was measured according to the determination of MLSS and assessed through the total number of carriers in a liter of reactor (Leyva-Díaz and Poyatos, 2015; Monteoliva-García et al., 2019).

For the analysis of PhACs, twelve influent and effluent wastewater 24-h composite samples were taken from sampling point 1 and 3 of the pilot-scale plant (Figure 1). The effluent samples were collected according to the constant hydraulic retention time (HRT) (12h). The first eight samples (influent and effluent) were retrieved at the beginning of the experimental period and after 35 days, both midweek and at the end of the weekend (02/08/2017, 06/08/2017 and 06/09/2017, 10/09/2017), the last four samples (influent and effluent) were taken at midweek after 35 days until the end of the experimental phase, (11/10/2017 and 15/11/2017). Every samples were taken using 500-mL amber PET bottles, as described previously (Gallardo-Altamirano et al., 2018).

2.3. Analytical methods for pharmaceutically active compounds

2.3.1. Chemical and sample treatment

All analytical reference standards were obtained from Sigma-Aldrich (St Louis, MO, USA), while the isotopically labeled compounds used as surrogates were purchased from Cerilliant (Round Rock, TX, USA) or CDN Isotopes (Quebec, Canada), or LGC Promochem (London, UK) or Santa Cruz Biotechnology (Dallas, TX, USA). All the above

compounds were prepared individually (100 µg mL-1) from the powder and dissolved in 100% acetonitrile (ACN) methanol (MeOH), or dimethyl sulfoxide (DMSO) according to the solubility of each compound and stored at −20 ° C. Working mixtures (10 µg L-1 in MeOH) for calibration and spiking purposes, including all tested compounds or labelled compounds were freshly prepared every three months. Their relevant physicochemical properties are reported elsewhere Gallardo-Altamirano et al. (2019) and (2018). LC-MS grade solvents (ACN ≥ 99.9%, MeOH ≥ 99.9%, DMSO ≥ 99.9%), and HPLC water were purchased from Merck (Darmstadt, Germany).

2.3.2. On-line extraction and LC-MS/MS analysis

Extraction of PhACs from wastewater samples were performed according to Gros et al., (2009) and López-Serna et al., (2010) and carefully detailed in Gallardo-Altamirano et al. (2018). Separation of the analytes was achieved on a Purospher STAR RP-18 endcapped column (125 x 2 mm i.d., 5 µm particle size, Merck, Darmstadt, Germany), while analysis was based on selective reaction monitoring (SRM) acquisition performed by a SCEX 4000 QTRAP hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer, equipped with a Turbo Ion Spray source (Sciex, Redwood City, CA, U.S.). Quantitative analysis was performed using the Analyst 1.5.2 Software (Sciex, Redwood City, CA, U.S.). Selected SRM transitions for each analyte and for its corresponding surrogate including the optimized parameters as well as any detailed information regarding LC-MS/MS methodology are described elsewhere (Gallardo-Altamirano et al. 2018, 2019).

2.4. Statistical analysis

Spearman`s rank correlation coefficients (ρ) were calculated to find significant links between the operational/environmental variables, organic matter and nutrient removal rate and REs of the selected 27 PhACs. Moreover, the Kruskal-Wallis non-parametric test was chosen to determinate significant differences among the different experimental phases and systems (conventional BNR A^2O systems vs A^2O-IFAS systems) using a 95% significant level (p <0.05).

3. Results and discussion

3.1. Operational parameters and evolution of biomass and physicochemical parameters of the pilot-scale A^2O-IFAS bioreactor

Figure 2 shows the evolution of suspended biomass (MLSS), attached biomass concentration (BFSS) and sludge volumetric index (SVI) during the start-up phase, stabilization phase and experimental phase of the A^2O-IFAS bioreactor. During the start-up phase (60 days) the MLSS was decreasing from 3500 mg L^{-1} to 1500 mg L^{-1} and the BFSS was increasing progressively from 0 mg L^{-1} to 1000 mg L^{-1} until the steady state was reached. The SVI was very high at the beginning (210-270 mL g^{-1}) producing several sedimentation problem episodes in the secondary settling tank. It was due to the introduction of the plastic carriers that disrupted the activate sludge flocs. Afterward, the sedimentation problems disappeared at the end of the start-up phase when the SVI decreased to 120 mL g^{-1} due to the restoration of the activated sludge flocs and the progressively decrease of MLSS concentration and ML-SRT (from 12 to 4 days); similar results were referenced by Kim et al. (2010). Subsequently, the stabilization phase started when steady-state conditions were achieved and the bioreactor operated at constant MLSS concentration (1500 ± 200 mg L^{-1}), constant BFSS (1000 ± 100 mg L^{-1}), constant SVI (120 ± 10 mL g^{-1}) and constant ML-SRT (4.0 ± 0.6 days) during 32 days. Finally, the experimental phase started at 2nd August with the first analysis of PhACs.

Table 2 shows the monthly average values for each operational parameter in the experimental phase. To find significant operational and performance advantages of the A^2O-IFAS system, the pilot-scale plant was overloaded, operating at peak hydraulic influent flowrate (251 L h^{-1} and 6.6 hours of HRT), instead of the design average hydraulic flowrate (167 L h^{-1}) and design HRT (10 hours). The F/M global ratio (MLSS+BFSS), % return activated sludge (RAS), HRT, ML-SRT and MLSS concentration values were kept constant during the experimental phase, whereas the % mixed liquor recycle (MLR) was changed from very high (310%) to low (116%) and medium (224%) during the experimental phase to observe the relationship with the total nitrogen removal rate (NRR) and N-NO_3^- concentration effluent. The attached biomass concentration (BFSS) increases progressively from 1299 ± 341 mg L^{-1} in August 2017 to 2152 ± 354 mg L^{-1} in November 2017 during the experimental phase (see Figure 2 and Table 2), in accordance with the constant increase of the organic loading rate (OLR) and nitrogen loading rate (NLR) (Table 2). The continuous increase of OLR with constant MLSS concentration created a gradually

Figure 2. Evolution of the biofilm fixed biomass (BFSS), the mixed liquor suspended solids (MLSS) and sludge vol
start-up, stabilization phase and experimental phase of the pilot-scaler A^2O-IFAS bioreactor.

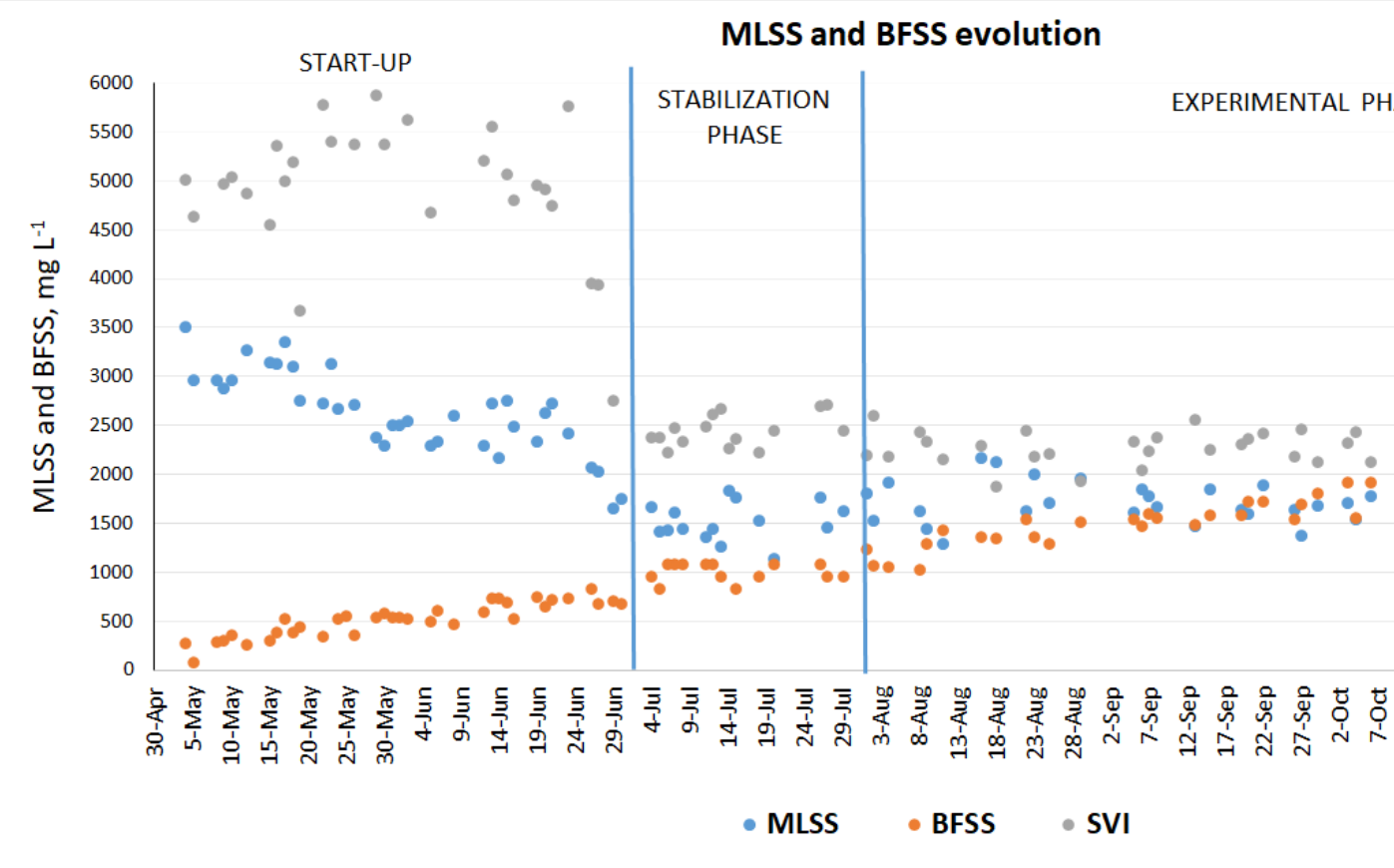


Table 2. Monthly average values and global average values (± standard deviation) for operational parameters of the A with an asterisk (*) were significantly different among each month, according to the Kruskal-Wallis test ($p < 0.05$).

Parameter	August 2017	September 2017	October 2017	01-15 No
Influent flow (L h^{-1})	246 ± 10	253 ± 16	258 ± 4	248 ± 5
HRT (h)	6.7 ± 0.3	6.6 ± 0.4	6.4 ± 0.1	6.7 ± 0.1
RAS (%)	47 ± 2	45 ± 3	42 ± 2.1	43 ± 1
MLR (%)	318 ± 13	307 ± 20	116 ± 13.2*	224 ± 4*
ML-SRT (d)	4.5 ± 0.6	3.5 ± 0.6	3.5 ± 0.2	4.4 ± 1.6
ML-F/M (kg BOD_5 kg MLVSS $^{-1}$ d^{-1})	0.37 ± 0.18	0.37 ± 0.21	0.40 ± 0.08	0.51 ± 0.
F/M global (kg BOD_5 kg biomass $^{-1}$ d^{-1})	0.20 ± 0.10	0.21 ± 0.10	0.23 ± 0.09	0.28 ± 0.
SVI (mL g^{-1})	112 ± 10	116 ± 7	108 ± 10.6	121 ± 15
DO set point (mg L^{-1})	0.5- 1.0	0.5- 1.0	0.5- 1.0	0.5- 1.0
MLSS (mg L^{-1})	1774 ± 274	1676 ± 155	1935 ± 275	1863 ± 1
MLVSS (%)	83 ± 7	85 ± 5	83 ± 7.13	85 ± 22
BFSS, mg/L	1299 ± 341*	1616 ± 184*	2003 ± 341*	2152 ± 3
BFVSS, %	82 ± 6	90 ± 3	88 ± 6.2	90 ± 5
OT (ºC)	28 ± 2	26 ± 1*	22 ± 2.2*	19 ± 1*
COD/TN ratio	6.0 ± 1.0	6.9 ± 0.8*	6.5 ± 0.9	7.1 ± 0.8
OLR (kg BOD_5 m^{-3} d^{-1})	0.564 ± 0.103	0.607 ± 0.249*	0.771 ± 0.134*	0.935 ± 0
ORR (%)	93.4 ± 2.0*	95.4 ± 1.4	96.0 ± 2.5	96.6 ± 0.
NLR (Kg TN m^{-3} d^{-1})	0.171 ± 0.028	0.161 ± 0.080	0.216 ± 0.021*	0.240 ± 0
NRR (%)	70.4 ± 3.3	80.6 ± 3.1*	70.6 ± 3.7	69.5 ± 4.
PLR (Kg TP m^{-3} d^{-1})	0.020 ± 0.001	0.021 ± 0.005	0.019 ± 0.001	0.020 ± 0
PRR (%)	47.7 ± 10.7*	82.0 ± 15.3	88.5 ± 10.4	81.9 ± 7.

HRT: hydraulic retention time; RAS: return activate sludge rate; MLR: mixed liquor recycle rate; ML-SRT: mixed l mixed liquor food-to-microorganisms ratio; SVI: sludge volumetric index; DO: dissolved oxygen; MLSS: mixed liq liquor volatile suspended solids; BFSS: biofilm suspended solid; BFVSS: biofilm volatile suspended solid; OT: operati rate; NLR: nitrogen loading rate; PLR: phosphorous loading rate; ORR: the corresponding Organic Removal Rate; Phosphorous Removal Rate. HRT, F/M, RAS, MLR, SRT, SVI, OLR, NLP and PRL were calculated as described by

increment of the ML-F/M ratio (from 0.37 to 0.51 kg BOD_5 kg MLVSS $^{-1}$ d^{-1}) that gave more assimilable organic matter for attached biomass growth. The operative temperature (OP) was decreasing from August (28ºC) to November (19ºC) while the dissolve oxygen was kept stable (0.5-1.0 mg L^{-1}). Slightly significant lower organic removal rate (ORR) (93.4 ± 2.0) and considerably significant lower phosphorous removal rate (PRR) (47.7 ± 10.7%) were obtained in August compared with the following months. This is possible due to the lower influent OLR (0.564 ± 0.103 kg BOD_5 m^{-3} d^{-1}), lower COD/TN ratio (6.0 ± 1.0) and lower BOD_5/TP ratio (28.2 ± 3.1) reported in August compared with the next months. These low values provide low easily biodegradable organic matter (BOD_5) for phosphorous accumulating microorganisms (PAOs) and denitrifying microorganisms. Additionally, some N-NO_3^- can be recycled by the RAS into the anaerobic zone and consume the (BOD_5) necessary for an efficient biological phosphorous removal process (Leyva-Díaz et al., 2016; Mannina et al., 2017; Saltnes et al., 2017). The NRR was similar during the all experimental phase except for September; the high value detected in this month was possibly due to the combination of lower NLR and higher OLR that produce higher COD/TN (6.9) and higher NRR (80.6 ± 3.1%). In this sense, higher nitrogen removal efficiencies have been reported at higher COD/TN ratio (Mannina et al., 2018, 2017; Regmi et al., 2011). Interestingly, similar NRR was obtained in August, October and November independently of the MLR rate (N-NO_3^- recycle ratio, responsible for the denitrification process) despite big variation was done during the experimental phase for each month. Therefore, independently of the MLR, a high percentage of total nitrogen removal occurred by simultaneous nitrification-denitrification process in the aerobic basin due to the combination of low oxygen dissolved (0.5-1.0 mg L^{-1}) and the presence of attached biomass that produces different redox conditions into the biofilm layer (Ashrafi et al., 2019; Iannacone et al., 2019).

3.2. Performance of the A^2O-IFAS pilot-scale plant: A^2O-IFAS system vs conventional A^2O system

Table 3 shows the mean concentration of COD, BOD_5, TSS, TN, N-NH_4^+, N-NO_3^-, TP, turbidity and pH in the bioreactor´s influent and effluent (point 2 and 3 respectively, Figure 1) during the experimental phase of the pilot-scale A^2O-IFAS plant. The mean effluent values were below the discharge limit value of the WWTP Murcia Este. Similar and better performance in terms of ORR, NRR and PRR was obtained in the present study compared with those studies that operated with analogous operational conditions (Araujo

Junior et al., 2013; Di Trapani et al., 2010; Kim et al., 2010; Mannina et al., 2018; Regmi et al., 2011), despite that the present A^2O-IFAS bioreactor was generally operated at lower HRT, higher NLR and lower COD/TN ratio. On the other hand, several authors obtained better organic and nutrients removal performance operating with IFAS-systems. In this sense, Xiao et al. (2016) obtained higher TN and TP removal (86% and 97%, respectively) in a full-scale A^2O-IFAS plant, and Ashrafi et al. (2019) obtained higher TN and TP removal (92.4% and 96.5%, respectively) in a pilot-scale IFAS five-stage Bardenpho plant. However, these bioreactors operated at considerable higher COD/TN ratio (10.33 and 14.2), higher HRT (11 and 8.4 hours, respectively) and lower NLR (0.096 and 0.100 Kg TN m^{-3} d^{-1}, respectively) compared with the present study (Table 2 and 3); in this sense, higher NRR and PRR have been related with bioreactor operated with higher anoxic and anaerobic HRT, higher COD/TN ratio and lower NLR (Barnard and Abraham, 2006; Mannina et al., 2018, 2017; Regmi et al., 2011; Tchobanoglous et al., 2003).

Table 3. Removal and average ± standard deviations of physical-chemical parameters measured in the decanted influent and effluent (point 2 and 3, Figure 1) water samples during the A^2O-IFAS system. TSS: Total suspended solids; TN: total N; TP: total P.

Parameter	Influent	Effluent	% Removal
COD (mg L^{-1})	376 ± 80	56 ± 13	**85.1 ± 2.9**
BOD_5 (mg L^{-1})	197 ± 47	8.8 ± 3.0	**95.5 ± 1.8**
TSS (mg L^{-1})	122 ± 33	15 ± 3.9	**87.6 ± 4.7**
TN (mg L^{-1})	57 ± 9.1	16 ± 5.2	**72.5 ± 6.3**
COD/TN (mg COD/mg TN)	6.6 ± 1.2		
N-NH_4^+ (mg L^{-1})	45 ± 8.3	1.1 ± 1.6	**97.6 ± 3.7**
N-NO_3^- (mg L^{-1})	0.2 ± 0.3	6.9 ± 2.3	
TP (mg L^{-1})	5.4 ± 0.4	1.3 ± 1.0	**76.6 ± 17.0**
Turbidity (NTU)	123 ± 30.8	7.2 ± 2.3	
pH	**7.4 ± 0.1**	**7.9 ± 0.1**	

Numerous comparisons have been done between IFAS-system and CAS processes to find the best performance of organic matter and nutrients removal with the lower operational cost. Generally, the IFAS-systems can operate with lower volume bioreactors, lower SRT and lower temperature than CAS systems to achieve a better nitrification-denitrification process. Di Trapani et al. (2013) demonstrated that high nitrification process was possible at low SRT and low temperatures. Accordingly, Araujo Junior et al. (2013) found that the addition of carriers (18% filling ratio) in a CAS process improved the NRR over 14% with a reduction of sludge waste production. Similarly, Güneş et al. (2019) found

in a comparison among CAS WWTP and a pilot-scale IFAS plant that higher NRR and PRR was possible in the IFAS-system with lower operational and investment costs (50%) and with a considerable lower orthophosphate effluent level (0.7 mg L^{-1} vs 4.2 mg L^{-1}). Moreover, Bashar et al. (2018) concluded in a study about the cost-effectiveness of TP removal efficiency that A^2O-IFAS system was one of the most cost-effective process ($42.22/lb-P removed) among six different process configurations for nutrient removal.

Tables S1 and S2 compare the environmental/operational parameters, physicochemical parameters and removal efficiency values achieved by the A^2O-IFAS bioreactor (phase III) with the values previously achieved by the A^2O bioreactor (Gallardo-Altamirano et al., 2018) in the same pilot-scale plant. Significant lower effluent concentration of BOD_5, total nitrogen (TN), total phosphorous (TP) and N-NO_3^- were obtained in this study (phase III, A^2O-IFAS system) compared with phase I (A^2O system), while significant lower effluent concentration of total suspended solid (TSS), N-NO_3^-, and turbidity were obtained in phase III (A^2O-IFAS system) compared with phase II (A^2O system) (Table S2). Consequently, significantly higher NRR (72.8 ± 4.4%) and PRR (75.0 ± 9.1%) were obtained in phase III compared with both phases operated with A^2O system (Table S1), while lower ORR were obtained only in phase I among phase II and III (Table S1). Interestingly, despite A^2O-IFAS bioreactor was operated at significant lower COD/TN rate compared with the A^2O bioreactor in phase II (6.6 ± 1.2 vs 7.8 ± 1.9, Table S2), higher nutrients removal efficiencies were achieved in the A^2O-IFAS bioreactor. Generally, higher biological nutrients removal efficiencies have been reported at higher COD/TN ratio (Mannina et al., 2018, 2017; Regmi et al., 2011). Therefore, this fact highlights the capacity of the attached biomass to enhance the TN and TP removal processes. Moreover, better performances were obtained by the A^2O-IFAS bioreactor compared to the A^2O bioreactor (phase I and II) despite the A^2O-IFAS system operated with significant lower RAS, ML-SRT, MLSS and HRT, as well as, significant higher ML-F/M ratio, NLR and PLR (Table S1) that normally decrease the organic and nutrients removal performance (Barnard and Abraham, 2006; Metcalf & Eddy, 2003). Operating with lower RAS, lower MLSS and lower HRT decrease the operational and investment cost of the WWTP (Bashar et al., 2018; Güneş et al., 2019). Additionally, operating with lower ML-SRT produces waste activated sludge with higher volatile biomass percentage (MLVSS of 84% vs 79%, Table S1) which increase the potential biodegradability and biogas production of the waste activated sludge in the further anaerobic digestion process (Bolzonella et al., 2005; Gonzalez et al., 2018; Xu et al., 2020). In addition, ML-SRT below 5 days is preferred for faster-growing

phosphorous organisms (PAOs) and other heterotrophs such as denitrifiers that improve the biological phosphorous and nitrogen removal capacity (Onnis-Hayden et al., 2011). Regarding settleability of the activated sludge, the A^2O-IFAS system shows significant higher SVI compared with phase I (114 ± 10 vs 84 ± 24 mL g^{-1}), while similar SVI were found with phase II. Despite this difference among phase I, the settling process was considered acceptable during the all experimental phases. Accordingly, Kim et al. (2010) demonstrated worse settling in A^2O-IFAS system than the control conventional A^2O system, although these differences were small and settling process was considered acceptable. On the other hand, Di Trapani et al. (2010) concluded that IFAS-systems improve the activated sludge settling process concerning CAS process.

Similar to the aforementioned authors, the present study demonstrated that IFAS-systems improve the CAS performance at lower operational and investment costs. Consequently, this process became a very simple and efficient technology for upgrading overloaded WWTPs or design a new municipal WWTP (di Biase et al., 2019; Leyva-Díaz et al., 2017).

3.3. Occurrence of PhACs in the influent and effluent wastewater samples

Table 4 display the range, mean, median, relative standard deviation (RSD) and frequency of the 27 targeted PhACs in the influent and effluent wastewater samples of the A^2O-IFAS system during the experimental phase. Only 3 compounds (sulfadiazine, sulfamethazine and paroxetine) had a frequency of detection <100% in the influent wastewater, while 7 compounds (acetaminophen, clarithromycin, sulfadiazine, sulfamethazine, metoprolol, fenofibrate, diazepam, and paroxetine) presented frequency of detection <100% in the effluent wastewater, in which all of them obtained very low mean concentration (<20 ng L^{-1}) included the AIAPs indomethacin and propyphenazone. Ibuprofen, acetaminophen and naproxen had the highest mean concentration (8716, 8667 and 5252 ng L^{-1}, respectively) in the influent wastewater samples, while the highest mean concentration in the effluent wastewater samples correspond for the antibiotic ofloxacin (2495 ng L^{-1}) and the diuretics hydrochlorothiazide (1748 ng L^{-1}) and furosemide (1034 ng L^{-1}). To our best knowledge, scarce studies exist about the PhACs concentration in the influents and effluents from pilot-scale or full-scale IFAS-system plants treating municipal wastewaters compared with CAS or MBR systems (De La Torre et al., 2015; Shreve and Brennan, 2019; Yadav et al., 2019). Interestingly, Shreve and Brennan, (2019) reported the

influent and effluent of 22 PhACs in 6 full-scale IFAS WWTPs, in which the average influent and effluent concentration of the targeted PhACs in the present study were in the

Table 4. Concentration range, mean concentration, median concentration, relative standard deviation (RSD) and det
active compounds (PhACs) in the influent wastewater samples of the A^2O-IFAS system. BLD: Below detection limit.

Therapeutic groups	Compounds	Influent (n=6)					Effluent (n=6	
		Range	Mean	Median	RSD (%)	Freq. (%)	Range	M
AIAPs	**Acetaminophen**	3174-14310	8667	9078	45	100	BLD -116	1
AIAPs	**Codeine**	111-253	210	232	25	100	87-179	1
AIAPs	**Diclofenac**	508-646	591	589	9	100	423-632	5
AIAPs	**Ibuprofen**	6600-11450	8716	8755	19	100	37-111	6
AIAPs	**Indomethacin**	11-18	14	14	19	100	10-19	1
AIAPs	**Ketoprofen**	910-1710	1236	1211	26	100	317-981	6
AIAPs	**Naproxen**	3663-6610	5252	5318	23	100	72-351	1
AIAPs	**Propyphenazone**	4.9-6.5	5.5	5.4	11	100	6-9	6
Antibiotics	**Clarithromycin**	197-529	321	268	44	100	BLD-23	1
Antibiotics	**Ofloxacin**	1448-3171	2467	2756	32	100	1679-3903	2
Antibiotics	**Sulfadiazine**	BLD	-	-	-	-	BLD	-
Antibiotics	**Sulfamethazine**	BLD-14	3.5	0.0	169	33	BLD-5	0
Antibiotics	**Sulfamethoxazole**	186-750	469	440	53	100	45-274	1
Antibiotics	**Trimethoprim**	102-398	219	203	51	100	6-97	5
Beta-blocker	**Atenolol**	581-953	754	750	18	100	14-261	1
Beta-blocker	**Metoprolol**	24-108	49	35	65	100	BLD-69	1
Beta-blocker	**Propranolol**	15-117	52	40	79	100	16-36	2
Beta-blocker	**Sotalol**	39-109	62	49	45	100	34-107	5
Diuretics	**Furosemide**	1134-1806	1475	1468	16	100	582-1401	1
Diuretics	**Hydrochlorothiazide**	1226-1917	1560	1547	15	100	1305-2169	1
Lipid-regulators	**Bezafibrate**	82-142	104	99	23	100	22-40	3
Lipid-regulators	**Fenofibrate**	7-74	48	54	56	100	BLD	-
Lipid-regulators	**Gemfibrozil**	651-1075	854	882	19	100	90-411	2
Psychiatrics	**Carbamazepine**	70-156	98	87	32	100	82-186	1
Psychiatrics	**Diazepam**	5-18	8.0	6.6	60	100	BLD-9	6
Psychiatrics	**Lorazepam**	101-305	158	142	48	100	125-320	1
Psychiatrics	**Paroxetine**	BLD -28	8.1	2.7	140	50	BLD -2	0

range of data reported by these authors. Generally, the range concentrations of all targeted PhACs in both influent and effluent wastewater was consistent with data from the reviewed studies (Luo et al., 2014; Tran et al., 2018; Verlicchi et al., 2012) that reported data mainly from CAS and MBR systems. However, the average concentration in the influent and effluent of the majority selected PhACs were below to the average influent and effluent concentrations reported by the review study Verlicchi et al. (2012).

Table S3 and S4 compare the mean concentration values in the influent and effluent wastewater samples of the A^2O-IFAS bioreactor (phase III) with the values previously achieved by the A^2O bioreactor (Gallardo-Altamirano et al., 2019, 2018) in the same pilot-scale plant. As shown in Table S3, 8 compounds (acetaminophen, diclofenac, ibuprofen, furosemide, hydrochlorothiazide, gemfibrozil, and carbamazepine) obtained significant lower influent concentration in phase III (A^2O-IFAS system) compared with phase I and II, while only diazepam and trimethoprim obtained higher significant influent concentration in phase III compared with both phases. Correspondingly, 11 compounds obtained significant lower effluent concentration in phase III compared with phase I and II (Table S4), while only sotalol and diazepam obtained significant higher effluent concentration in phase III compared with phase I and II. The significant lower effluent concentration for many compounds in phase III (A^2O-IFAS system) was due to the significant lower influent concentration for several compounds in phase III, as well as, for the significant higher REs observed in A^2O-IFAS system, as it will be further discussed in Section 3.4.

In order to identify which compound could pose a risk for aquatic ecosystems, the lowest predict non-effect concentration (PNEC) values described in the recent literature (Orias and Perrodin, 2013; Tran et al., 2018; Verlicchi et al., 2012) for all tested PhACs are shown in Table S4. Out of the 27 targeted PhACs, seven compounds had mean effluent concentration higher than their PNEC (diclofenac, ibuprofen, ofloxacin, sulfamethoxazole, trimethoprim, furosemide and gemfibrozil) in the A^2O-IFAS system. Therefore, these compounds could pose a risk for the aquatic ecosystems (Tran et al., 2018; Verlicchi et al., 2012). However, the number of compounds with higher mean effluent concentration than their PNEC were almost double (12) in phase I and II (A^2O system). Consequently, the application of the IFAS system to the A^2O bioreactor could reduce considerably the risk for the aquatic ecosystem (from 12 to 7 compounds).

3.4. Removal efficiency of PhACs and links with the operational/performance variables: A^2O-IFAS system vs conventional A^2O system

Table 5 shows the REs (range, mean, median and RSD) of the targeted PhACs throughout the experimental phase in the A^2O-IFAS system. Those pharmaceuticals with mean influent concentration <20 ng L^{-1} and influent frequency detection <50% (indomethacin, propyphenazone, sulfadiazine, sulfamethazine, diazepam and paroxetine) were omitted from the calculations, since conclusions concerning its REs could not be accurately drawn.

The highest average REs values (>80%) correspond for fenofibrate, acetaminophen, ibuprofen, naproxen, clarithromycin and atenolol, highlighting that those PhACs obtained the lowest RSD percentages values (from 0% to 14%). The removal of the majority targeted PhACs are mainly attributed to the biodegradation/biotransformation process in the secondary biological treatment (Ashfaq et al., 2017; Gao et al., 2012; Grandclément et al., 2017; Jelić et al., 2012; Verlicchi et al., 2012; Zorita et al., 2009). However, the high RE of fenofibrate was attributed to sorption mechanisms due to the high lipophilicity properties indicated by the high coefficient octanol-water (K_{ow}=5.19) (Jelić et al., 2011; Verlicchi et al., 2012). On the other hand, very low and negative average REs values (<30%) were obtained for lorazepam, carbamazepine, ofloxacin, hydrochlorothiazide, sotalol, diclofenac, propranolol and metoprolol, highlighting that those PhACs obtained the highest RSD. Negative REs of the aforementioned PhACs are commonly observed and reported in wastewater treatment plants operated with different treatment process (CAS, MBR, MBBR and IFAS systems) and are likely due to the release of molecules enclosed/absorbed in suspended particles or due to the microbially-mediated reversion of influent metabolites conjugate forms into the parent compounds (Ashfaq et al., 2017; Grandclément et al., 2017; Luo et al., 2014; Shreve and Brennan, 2019; Verlicchi et al., 2012). The remaining PhACs obtained medium RE values (between 30% to 70%) with medium RSD percentage values (between 11% and 58%). In general, the average RE values obtained in the A^2O-IFAS system for the selected PhACs are in the range of REs reported by different studies operating with MBBR or IFAS systems, except for diclofenac that was lower compared with the REs values reported in recent studies (De La Torre et al., 2015; Luo et al., 2015; Ooi et al., 2018; Shreve and Brennan, 2019) and for clarithromycin and atenolol for which it was higher (Casas et al., 2015; Escolà Casas et al., 2015; Falås et al., 2013; Ooi et al., 2018). In accordance to the best of author's knowledge, no REs data in literature for

continuous MBBR or IFAS process have been reported for ofloxacin, sotalol, furosemide and lorazepam.

Table 5. Removal efficiencies (REs, %) of PhACs and relative standard deviations (RSD) calculated for each compound measured in the A^2O-IFAS system. The compounds with average values < 20 ng/L and a frequency detection <50% are not shown.

Therapeutic groups	Compounds	Range	Mean	Median	RSD (%)	Freq. (%)
AIAPs	**Acetaminophen**	99-100	99.8	100	0.41	100
AIAPs	**Codeine**	18-63	33	29	50	100
AIAPs	**Diclofenac**	-10-23	9.2	8.5	122	100
AIAPs	**Ibuprofen**	99-100	99.5	100	1	100
AIAPs	**Ketoprofen**	39-65	51	50	19	100
AIAPs	**Naproxen**	94-98	97	98	2	100
Antibiotics	**Clarithromycin**	90-100	96	97	5	100
Antibiotics	**Ofloxacin**	-170-39	-17	11	-468	100
Antibiotics	**Sulfamethoxazole**	29-77	63	69	30	100
Antibiotics	**Trimethoprim**	37-98	69	71	39	100
Beta-blocker	**Atenolol**	63-98	84	86	14	100
Beta-blocker	**Metoprolol**	-193-100	29	64	381	100
Beta-blocker	**Propranolol**	-43-79	22	30	261	100
Beta-blocker	**Sotalol**	-5-30	8.8	2.5	156	100
Diuretics	**Furosemide**	8-58	30	28	58	100
Diuretics	**Hydrochlorothiazide**	-28-9	-12	-15	-122	100
Lipid-regulators	**Bezafibrate**	55-76	69	72	11	100
Lipid-regulators	**Fenofibrate**	100	100.0	100	0	100
Lipid-regulators	**Gemfibrozil**	56-86	70	71	16	100
Psychiatrics	**Carbamazepine**	-30--9	-19	-18	-37	100
Psychiatrics	**Lorazepam**	-71--5	-25	-16	-103	100

During the last decades, many studies have compared the REs of PhACs by the most common technology for wastewater treatment, CAS and MBR systems (Clara et al., 2004; Kreuzinger et al., 2004; Park et al., 2017; Petrovic et al., 2009; Radjenovic et al., 2007). However, few studies have compared the REs of PhACs by these conventional technologies versus MBBR or IFAS systems (De La Torre et al., 2015; Shreve and Brennan, 2019). In this sense, De La Torre et al. (2015) compared the REs of several PhACs in a full-scale CAS plant with a semi-real plant operated with different configuration (MBR, IFAS-MBR, pure MBBR) that treated the same urban wastewater. They concluded that the IFAS-MBR system exhibited similar or better REs for most of the studied PhACs, in which the operating conditions (SRT, MLSS, HRT and F/M ratio) were proved to be important because lower removal rates were obtained at lower SRT and lower MLSS concentration. A similar comparison between technologies was done in bench-scale experiments; for instance, Murray et al. (2017) found in parallel bench-scale sequencing

batch reactors fed with real municipal wastewater higher REs of atenolol and trimethoprim in the IFAS reactor compared to the control CAS reactor. Likewise, Falås et al. (2013), (2012) and Jewell et al. (2016) compared the IFAS system with the CAS system through bench-scale batch experiments using both activated sludge and suspended biofilm carrier from full-scale IFAS WWTPs. Their results proved that attached biomass contributed significantly to the removal of some PhACs in the IFAS processes. However, more research is needed to explore the higher RE capacity of these technologies and links to the operational/performance variables.

Table S5 shows the mean ± standard deviation of the REs values obtained in the A^2O-IFAS bioreactor (phase III) with the REs values obtained in the conventional A^2O bioreactor previously studied in two experimental phases (phase I and II) by Gallardo-Altamirano et al. (2018). Additionally, in order to find significant links among the RE obtained in the three phases with its corresponding operational/performance parameters, the Spearman's rank correlation coefficient (ρ) was calculated in Table S6. The SRT, MLSS and BFSS operational parameters was no included in the test because the A^2O-IFAS systems operate with two types of biomass (suspended activated sludge and attached biomass) where the attached-biomass SRT is considered higher than the ML-SRT. In this sense, those variables are not equally comparable among the two different technologies (A^2O vs A^2O-IFAS system) because the microbial diversity and function of the biomass is different and enhanced in the A^2O-IFAS system (Shreve and Brennan, 2019).

According to the Kruskal-Wallis test, statistically significant higher RE was observed for phase III (A^2O-IFAS system) compared with phase I and II (A^2O system) for ibuprofen, naproxen and trimethoprim (Table S5). The REs of the AIAPs ibuprofen and naproxen were low in phase I (39% and 41%, respectively), high in phase II (88% and 87%) and very high in phase III (99.5% and 97%). The increase of the RE between phase I and II was favored by the increase of MLSS and the decrease of F/M ratio according to Gallardo-Altamirano et al. (2018). Similarly, the significant increase of RE in phase III compared with phase I and phase II was favored by the decrease of the global F/M ratio for ibuprofen (ρ=-0.62, Table S6) and naproxen (ρ=-0.52, Table S6), as well as the increase of the operating temperature (OT, ρ=0.53) and NRR (ρ=0.53) for naproxen. Concerning to the antibiotic trimethoprim, no significant RE were found between phase I and II (Gallardo-Altamirano et al., 2019), while significant higher RE (Table S5) were obtained in phase III with the presence of attached biomass and favored by higher OT (ρ=0.56). Similarly, Murray et al. (2017) demonstrated improved RE of trimethoprim under all conditions in

the IFAS bioreactors as compared to the CAS control bioreactors, also correlated with higher OT. In this sense, Falås et al. (2013) also reported considerable higher removal rate of trimethoprim in a bath experiment using suspended biofilm carrier compared with activated sludge. The attached biomass systems can lead to different redox conditions at different thicknesses of the biofilm layer that may enhance the removal of several micropollutants (Grandclément et al., 2017; Torresi et al., 2016). In this sense, several authors have reported high REs of naproxen and trimethoprim in anaerobic conditions (Alvarino et al., 2016; Gonzalez-Gil et al., 2019, 2016; Yang et al., 2017). Therefore, higher RE of trimethoprim and naproxen in the A^2O-IFAS system may be related also to the higher anaerobic conditions produce by the attached biomass.

Furthermore, statistically significant higher RE was observed for phase III (A^2O-IFAS system) and phase II compared with phase I (A^2O system) for ketoprofen, gemfibrozil, atenolol, clarithromycin and bezafibrate (Table S5). Interestingly, these higher RE in phase II and III was favored for the decrease of global F/M ratio (Table S6). Accordingly, several authors (De La Torre et al., 2015; Falås et al., 2012; Luo et al., 2015) found in parallel studies considerable higher RE of ketoprofen and gemfibrozil in IFAS process compared to CAS and MBR systems. They concluded that the IFAS process shown higher RE for most of the studied PhACs, highlighting the importance of operate at high SRT and MLSS concentration that give low F/M ratio to increase the RE of several PhACs (De La Torre et al., 2015). Similarly, Ooi et al. (2018) found high RE of atenolol (79%) and clarithromycin (78%) in a pilot-scale staged anoxic/aerobic MBBR system. The nitrifying basin of the pilot-scale plant obtained higher biodegradation rate per gram of biomass for atenolol, while the denitrifying basin obtained higher biodegradation rate for clarithromycin, it pointed out the importance of the denitrification process (shown in the NRR value) for the biodegradation/transformation of clarithromycin. Equally, a strong positive correlation of the RE values with NRR and influent $N\text{-}NH_4^+$ concentration for clarithromycin (ρ=0.62 and ρ=0.55, respectively) was found in our study. Moreover, Murray et al. (2017) also found higher RE of atenolol in IFAS process compared with the control CAS. Finally, similar to our study, Falås et al. (2013) also found higher removal rate per gram of biomass for bezafibrate in the suspended biofilm carriers compared to suspended activated sludge; interestingly, the higher RE of bezafibrate in IFAS system was positivity correlated in our study (Table S6) with the $N\text{-}NH_4^+$ influent concentration, ORR, NRR, and PRR (ρ=0.67, ρ=0.59, ρ=0.59, ρ=0.44, respectively)

Lastly, the beta-blocker sotalol and the AIAPs diclofenac showed significant higher RE in phase III compared with phase II but the RE was very low (8.8% and 9.2% respectively). Correspondingly, low RE (<40%) was also reported for sotalol in bath experiment for IFAS or MBBR systems by several studies (Casas et al., 2015; Escolà Casas et al., 2015; Ooi et al., 2018). However, contrary to the present study, higher RE (>30%) was normally obtained for diclofenac in IFAS or MBBR system in the literature (De La Torre et al., 2015; Falås et al., 2012; Jewell et al., 2016; Luo et al., 2015; Ooi et al., 2018; Shreve and Brennan, 2019). On the other hand, only the diuretic hydrochlorothiazide obtained significant lower mean RE in phase III compared with phase I (-12% vs 33%) (Table S5). Similarly, low RE values (<2%) was obtained in full-scale IFAS WWTP and batch experiment with attached biomass and activated sludge by Falås et al. (2013).

To conclude, the aforementioned studies determined that IFAS or MBBR systems reach similar or better RE of PhACs compared to other technologies, where the biodegradation generally occurred in parallel to the removal of organic matter, nitrogen and phosphorous indicating co-metabolism (Casas et al., 2015; Grandclément et al., 2017; Ooi et al., 2018) as it occurred with naproxen, clarithromycin and bezafibrate in the present study. Additionally, the combination of suspended activated sludge and attached-growth biomass in the A^2O-IFAS bioreactor gives a combination of slow-growing microorganisms in the carrier media and quick-growing microorganisms in the activated sludge that increases the total biomass concentration (MLSS + BFSS) and the global SRT. Therefore, the bioreactor operates at lower global F/M ratio with a high range of possible active strains capable of improve the biodegradation/biotransformation rate of several PhACs and organic micropollutant (Casas et al., 2015; Escolà Casas et al., 2015; Falås et al., 2013; Grandclément et al., 2017; Leyva-Díaz et al., 2020; Polesel et al., 2017; Shreve and Brennan, 2019). Finally, due to the affordable cost and well-demonstrated high efficiency in organic matter, nutrients and organic micropollutants removal, IFAS systems became one of the most promising technologies for conventional WWTP upgrading.

4. Conclusion

The A^2O-IFAS system showed similar or better performance in terms of ORR, NRR and PRR compared with the conventional A^2O systems. In particular, significantly higher NRR (72.8 ± 4.4%) and PRR (75.0 ± 9.1%) were obtained compared with A^2O system. Despite A^2O-IFAS bioreactor was operated at significant lower COD/TN rate compared with the A^2O bioreactor (6.6 ± 1.2 vs 7.8 ± 1.9). This fact highlights the capacity of the

attached biomass to enhance the TN and TP removal processes. Besides, the A^2O-IFAS system operated with significant lower RAS, ML-SRT, MLSS and HRT, as well as, significant higher ML-F/M ratio, NLR and PLR, that normally decrease the organic and nutrients removal performance. All these aspects imply lower operational and investment costs.

In relation to PhACs removal, previous studies showed that IFAS process achieves higher RE for most of the studied PhACs compared to CAS process, highlighting the importance of operate at high SRT and MLSS concentration that give low F/M ratio to increase the RE of several PhACs. In the A^2O-IFAS system, the combination of suspended activated sludge and attached-growth biomass gives a higher total biomass concentration (MLSS + BFSS). This implies a similar or better RE of PhACs, operating at very low values of ML-SRT (4.0 days) and MLSS (1822 mg/L). The highest average REs values (>80%) correspond for fenofibrate, acetaminophen, ibuprofen, naproxen, clarithromycin and atenolol. Very low and negative average REs values (<30%) were obtained for lorazepam, carbamazepine, ofloxacin, hydrochlorothiazide, sotalol, diclofenac, propranolol and metoprolol. The remaining PhACs obtained medium RE values (between 30% to 70%).

According to these results, due to the affordable cost and well-demonstrated high efficiency in organic matter, nutrients and organic micropollutants removal, IFAS systems become one of the most promising technologies for conventional WWTP upgrading.

Supplementary material for Chapter 4:

"**Fate of pharmaceutically active compounds in a pilot-scale A^2O integrated fixed-film activated sludge (IFAS) process treating municipal wastewater"**

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Table S1. Average values (± standard deviation) for operational parameters of the three phases operated with a conve
A^2O-IFAS system. Parameters marked with an asterisk (*) were significantly different between the experimental phas
an "x" were significantly different between the experimental phases II and III, according to the Kruskal-Wallis test ($p <$
were reported by (Gallardo-Altamirano et al., 2018).

Parameter	Phase I (A^2O)	Phase II (A^2O)	I
Influent flow (L h^{-1})	237 ± 23	243 ± 32	2
HRT (h) *	7.0 ± 0.7	6.8 ± 0.9	6
RAS (%) * x	58 ± 6	74 ± 10	4
MLR (%)	170 ± 39	209 ± 27	2
ML-SRT (d) * x	12.4 ± 4.4	13.1 ± 2.8	4
ML-F/M (kg BOD_5 kg MLVSS $^{-1}$ d^{-1}) * x	0.34 ± 0.14	0.21 ± 0.06	0
F/M global (kg BOD_5 kg MLVSS $^{-1}$ d^{-1}) *	0.34 ± 0.14	0.21 ± 0.06	0
SVI (mL g^{-1}) *	84 ± 24	106 ± 22	1
DO set point (mg L^{-1})	0.5 - 2.0	0.5- 2.0	0
MLSS (mg L^{-1}) * x	2663 ± 434	5079 ± 529	1
MLVSS (%) * x	79 ± 4	79 ± 3	8
BFSS, mg/L	-	-	1
BFVSS, %	-	-	8
OT (ºC) x	24.3 ± 2.9	17.2 ± 2.5	2
OLR (kg BOD_5 m^{-3} d^{-1}) x	0.625 ± 0.173	0.851 ± 0.272	0
ORR (%) *	91.6 ± 4.0	95.6 ± 2.0	9
NLR (Kg TN m^{-3} d^{-1}) *	0.181 ± 0.020	0.194 ± 0.023	0
NRR (%) * x	56.6 ± 10.7	67.5 ± 3.9	7
PLR (Kg TP m^{-3} d^{-1}) *	0.017 ± 0.004	0.018 ± 0.001	0
PRR (%) * x	49.1 ± 13.2	66.2 ± 13.1	7

Table S2. Removal and average ± standard deviations of physical-chemical parameters measured in influent and efflue
for the A^2O system and phase III for the A^2O-IFAS system. Data marked with an asterisk (*) are significantly different
according to the Kruskal-Wallis test ($p<0.05$). COD: chemical oxygen demand; BOD_5: biological oxygen demand at 5
total N; TP: total P. The data shown in Phase I and II were reported by (Gallardo-Altamirano et al., 2018).

	COD	**BOD_5**	**TSS**	**TN**	**COD/TN**	**$N\text{-}NH_4^+$**	**$N\text{-}NO_3^-$**	**TI**
Influent, mg L^{-1}								
Phase I	338 ± 57	189 ± 43	104 ± 30*	53.4 ± 10.5	6.5 ± 1.1	40.2 ± 7.5	0.3 ± 0.3	4.8
Phase II	424 ± 103*	234 ± 78*	145 ± 47*	51.2 ± 10.6	7.8 ± 1.9	41.5 ± 12.5	0.2 ± 0.4	5.1
Phase III	376 ± 80*	197 ± 47*	122 ± 33*	57 ± 9.1	6.6 ± 1.2 x	45 ± 8.3	0 ± 0.3	5.4
Effluent, mg L^{-1}								
Phase I	54 ± 14	14 ± 6*	15 ± 4.6	22.2± 4.8*	-	1.6 ± 2.4	10.6 ± 3.7 *	2.5
Phase II	52 ± 15	9.4 ± 3.8	21 ± 7.5*	18.3 ± 3.4	-	1.0 ± 3.1	11.1 ± 4.0 *	1.7
Phase III	56 ± 13	8.8 ± 3.0*	15 ± 3.9*	16 ± 5.2*	-	1.1 ± 1.6	6.9 ± 2.3*	1.3

Table S3. Concentration (ng/L) of the 27 PhACs in the influent wastewater samples for the experimental phases I (n= phase III (n=6) for the A^2O-IFAS system. Values shown are means ± SD; detection frequencies of each compound are an asterisk (*) were significantly different between the experimental phases I and III, and PhACs marked with an "x" experimental phases II and III, according to the Kruskal-Wallis test ($p < 0.05$). BLD. Below detection limit. The data sl (Gallardo-Altamirano et al., 2018, 2019).

Therapeutic groups	Compounds	Phase I (A^2O)	Phase II (A^2O)
AIAPs	**Acetaminophen***	163475 ± 152899 (100)	110942 ± 122426 (100)
AIAPs	**Codeine**	192 ± 159 (75)	195 ± 149 (100)
AIAPs	**Diclofenac* x**	1293 ± 294 (100)	1093 ± 198 (100)
AIAPs	**Ibuprofen* x**	15970 ± 6924 (100)	20000 ± 4067 (100)
AIAPs	**Indomethacin**	21 ± 29 (75)	28 ± 27 (100)
AIAPs	**Ketoprofen**	1041 ± 253 (100)	1288 ± 243 (100)
AIAPs	**Naproxen**	7753 ± 3578 (100)	7264 ± 2802 (100)
AIAPs	**Propyphenazone**	4.4 ± 7.8 (50)	6.4 ± 3.3 (100)
Antibiotics	**Clarithromycin**	447 ± 365 (100)	784 ± 311 (100)
Antibiotics	**Ofloxacin**	2610 ± 2077 (100)	48494 ± 63454 (100)
Antibiotics	**Sulfadiazine**	16 ± 18 (75)	1.5 ± 2.9 (40)
Antibiotics	**Sulfamethazine**	BLD	1.2 ± 2.5 (40)
Antibiotics	**Sulfamethoxazole**	218 ± 437 (25)	216 ± 299 (40)
Antibiotics	**Trimethoprim***	92 ± 36 (100)	119 ± 36 (100)
Beta-blocker	**Atenolol**	635 ± 363 (100)	611 ± 216 (100)
Beta-blocker	**Metoprolol**	BLD	6.8 ± 9.3 (40)
Beta-blocker	**Propranolol**	136 ± 197 (75)	35 ± 43 (100)
Beta-blocker	**Sotalol**	41 ± 21 (100)	38 ± 8.0 (100)
Diuretics	**Furosemide* x**	3215 ± 1047 (100)	2792 ± 1179 (100)
Diuretics	**Hydrochlorothiazide* x**	2790 ± 575 (100)	2640 ± 524 (100)
Lipid-regulators	**Bezafibrate**	127 ± 52 (100)	139 ± 49 (100)
Lipid-regulators	**Fenofibrate**	BLD	BLD
Lipid-regulators	**Gemfibrozil* x**	1625 ± 532 (100)	1426 ± 448 (100)
Psychiatrics	**Carbamazepine***	154 ± 63 (100)	98 ± 20 (100)
Psychiatrics	**Diazepam* x**	0.5 ± 1.1 (25)	0.3 ± 0.5 (40)
Psychiatrics	**Lorazepam**	241 ± 83 (100)	182 ± 46 (100)
Psychiatrics	**Paroxetine**	4.8 ± 5.7 (50)	2.2 ± 4.8 (20)

Table S4. Concentration (ng/L) of the 27 PhACs in the effluent wastewater samples for the experimental phases I (n= phase III (n=6) for the A^2O-IFAS system. Values shown are means ± SD; detection frequencies of each compound are an asterisk (*) were significantly different between the experimental phases I and III, and data marked with an "x" v experimental phases II and III, according to the Kruskal-Wallis test ($p < 0.05$). Data boldfaced are higher than the mi (PNEC). BLD. Below detection limit. The data shown in Phase I and II were reported by (Gallardo-Altamirano et al., 2

Therapeutic groups	Compounds	Phase I (A^2O)	Phase II (A^2O)	Phase III (A^2O
AIAPs	**Acetaminophen* x**	**6226** ± 10212 (75)	**1234** ± 2354 (100)	19 ± 47 (17)
AIAPs	**Codeine**	80 ± 160 (25)	146 ± 146 (80)	137 ± 40 (100)
AIAPs	**Diclofenac* x**	**1142** ± 196 (100)	**1266** ± 213 (100)	**538** ± 86 (100)
AIAPs	**Ibuprofen* x**	**8795** ± 2239 (100)	**1638** ± 3508 (60)	**62** ± 28 (100)
AIAPs	**Indomethacin**	7.7 ± 7.3 (75)	5.2 ± 3.5 (80)	14 ± 3.7 (100)
AIAPs	**Ketoprofen**	1151 ± 572 (100)	737 ± 411 (100)	624 ± 243 (100
AIAPs	**Naproxen***	**4003** ± 1516 (100)	**938** ± 1107 (100)	191 ± 127 (100
AIAPs	**Propyphenazone**	0.17 ± 0 (25)	5.2 ± 4.6 (80)	6.9 ± 1.1 (100)
Antibiotics	**Clarithromycin* x**	**168** ± 60 (100)	**55** ± 97 (60)	10 ± 11 (68)
Antibiotics	**Ofloxacin**	**2075** ± 2249 (100)	**51636** ± 68801 (100)	**2495** ± 865 (10
Antibiotics	**Sulfadiazine* x**	64 ± 122 (50)	0.7 ± 1.6 (20)	BLD
Antibiotics	**Sulfamethazine**	BLD	BLD	0.8 ± 1.8 (17)
Antibiotics	**Sulfamethoxazole**	**93** ± 186 (25)	**115** ± 166 (40)	**162** ± 84 (100)
Antibiotics	**Trimethoprim x**	**67** ± 48 (100)	**105** ± 17 (100)	**50** ± 42 (100)
Beta-blocker	**Atenolol**	262 ± 203 (100)	110 ± 48 (100)	118 ± 84 (100)
Beta-blocker	**Metoprolol**	BLD	3.0 ± 4.2 (40)	18 ± 26 (67)
Beta-blocker	**Propranolol**	**119** ± 226 (75)	**140** ± 247 (60)	23 ± 7.3 (100)
Beta-blocker	**Sotalol***	28 ± 15 (100)	43 ± 7.4 (100)	56 ± 27 (100)
Diuretics	**Furosemide* x**	**2323** ± 182 (100)	**2184** ± 592 (100)	**1034** ± 285 (10
Diuretics	**Hydrochlorothiazide x**	1714 ± 741 (100)	2646 ± 507 (100)	1748 ± 328 (10
Lipid-regulators	**Bezafibrate***	81 ± 28 (100)	26 ± 20 (80)	31 ± 6.8 (100)
Lipid-regulators	**Fenofibrate**	BLD	BLD	BLD
Lipid-regulators	**Gemfibrozil* x**	**1085** ± 216 (100)	**377** ± 247 (100)	**268** ± 123 (100
Psychiatrics	**Carbamazepine**	127 ± 29 (100)	114 ± 20 (100)	117 ± 38 (100)

Psychiatrics	**Diazepam* x**	BLD	1.4 ± 2.2 (40)	6.2 ± 3.1 (83)
Psychiatrics	**Lorazepam**	**216** ± 47 (100)	**194** ± 36 (100)	188 ± 67 (100)
Psychiatrics	**Paroxetine**	BLD	BLD	0.3 ± 0.6 (17)

[a] Verlicchi et al. (2012)
[b] Orias and Perrodin (2013)
[c] Tran et al. (2018)

Table S5. Removal efficiencies (REs, %) of PhACs for the experimental phases I (n=4) and II (n=5) for the A^2O systen system. Values shown are means ± SD; detection frequencies in the influent of each compound are shown in brackets. I significantly different between the experimental phases I and III, and data marked with an "x" were significantly diffe and III, according to the Kruskal-Wallis test ($p < 0.05$). The compounds with average concentration influent values < 2 are not shown. The data shown in Phase I and II were reported by (Gallardo-Altamirano et al., 2018, 2019).

Therapeutic groups	Compounds	Phase I (A^2O)	Phase II (A^2O)
AIAPs	**Acetaminophen**	94 ± 8 (100)	100 ± 0.9 (100)
AIAPs	**Codeine**	32 ± 117 (75)	42 ± 36 (100)
AIAPs	**Diclofenac x**	7.3 ± 31 (100)	-18 ± 23 (100)
AIAPs	**Ibuprofen* x**	39 ± 23 (100)	88 ± 25 (100)
AIAPs	**Ketoprofen***	-13 ± 62 (100)	39 ± 38 (100)
AIAPs	**Naproxen* x**	41 ± 32 (100)	87 ± 16 (100)
Antibiotics	**Clarithromycin***	30 ± 57 (100)	93 ± 11 (100)
Antibiotics	**Ofloxacin**	-42 ± 187 (100)	18 ± 24 (100)
Antibiotics	**Sulfamethoxazole**	-	-
Antibiotics	**Trimethoprim* x**	30 ± 37 (100)	7.8 ± 18 (100)
Beta-blocker	**Atenolol****	48 ± 52 (100)	81 ± 9.5 (100)
Beta-blocker	**Metoprolol**	-	-
Beta-blocker	**Propranolol**	-90 ± 341 (100)	-100 ± 271 (100)
Beta-blocker	**Sotalol x**	28 ± 27 (100)	-19 ± 26 (100)
Diuretics	**Furosemide**	22 ± 24 (100)	15 ± 20 (100)
Diuretics	**Hydrochlorothiazide***	38 ± 29 (100)	-1.0 ± 12 (100)
Lipid-regulators	**Bezafibrate***	33 ± 22 (100)	80 ± 17 (100)
Lipid-regulators	**Fenofibrate**	-	-
Lipid-regulators	**Gemfibrozil***	31 ± 13 (100)	74 ± 17 (100)
Psychiatrics	**Carbamazepine**	4.8 ± 42 (100)	-18 ± 10 (100)
Psychiatrics	**Lorazepam**	1.3 ± 43 (100)	-8.2 ± 9.0 (100)

Table S6. Spearman rank-correlation coefficients (ρ) between the removal efficiencies (%) and the operational/per corresponding to experimental phase I and II reported by Gallardo-Altamirano et al. (2019, 2018) and the IFAS-A boldfaced in green and red (red: p<0.05; green: p < 0.10).

Compounds	global F/M	OT	BOD_5 influent	COD influent	TN influent	$N\text{-}NH_4^+$ influent
AIAPs						
Acetaminophen	**-0.47**	0.39	-0.40	-0.18	-0.22	0.08
Codeine	0.16	0.22	-0.23	-0.36	**-0.50**	-0.16
Diclofenac x	0.17	0.22	-0.32	-0.34	-0.08	-0.21
Ibuprofen* x	**-0.62**	0.19	-0.35	-0.07	-0.09	0.28
Ketoprofen*	**-0.46**	0.34	-0.33	-0.21	-0.36	0.18
Naproxen*x	**-0.52**	**0.53**	**-0.55**	-0.21	-0.10	0.33
Antibiotics						
Clarithromycin*	**-0.67**	0.14	-0.29	0.05	0.01	**0.55**
Ofloxacin	0.07	0.03	-0.24	-0.31	-0.36	-0.15
Sulfamethoxazole	-0.01	-0.25	0.21	-0.13	0.25	0.04
Trimethoprim* x	-0.25	**0.56**	**-0.64**	**-0.59**	-0.20	-0.07
Beta-blocker						
Atenolol **	**-0.46**	0.19	-0.40	-0.09	-0.02	0.14
Propranolol	-0.17	0.10	-0.30	-0.42	-0.31	-0.10
Sotalol x	0.26	0.38	**-0.45**	**-0.63**	-0.26	**-0.49**
Diuretics						
Furosemide	0.24	-0.13	0.10	0.24	**0.48**	0.18
Hydrochlorothiazide*	**0.69**	-0.06	0.21	-0.16	-0.28	**-0.46**
Lipid-regulators						
Bezafibrate*	**-0.51**	-0.17	0.01	0.17	0.03	**0.67**
Gemfibrozil*	**-0.53**	0.18	-0.26	-0.03	-0.28	0.38
Psychiatrics						
Carbamazepine	0.37	-0.21	0.22	0.22	0.24	-0.08
Lorazepam	0.32	**-0.53**	**0.48**	0.23	0.12	0.07

F/M global (kg BOD_5 kg biomass^{-1} d^{-1}): food-to-microorganisms ratio; OT (ºC): operating temperature; OLR (kg
NLR (kg TN m^{-3} d^{-1}): nitrogen loading rate; PLR (kg TP m^{-3} d^{-1}): phosphorous loading rate; ORR (%): Organic Remo
Rate; PRR (%): Phosphorous Removal Rate HRT, F/M, RAS, MLR, SRT, SVI, OLR, NLP and PRL were calcul

References

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V. DISCUSIÓN GENERAL

Durante las últimas décadas, la producción de aguas residuales ricas en nutrientes ha ido incrementándose continuamente en todo el mundo debido al continuo crecimiento de las ciudades junto con un mayor consumo en los hogares y a una mayor producción industrial (EEA, 2018). Además, debido a una mayor sensibilidad de los métodos analíticos y a la mejora continua de la instrumentación analítica, se ha podido observar una continua acumulación de diferentes micro contaminantes orgánicos (p.ej. productos de higiene y cuidados corporal, filtros ultravioletas, fragancias sintéticas y más concretamente los compuestos farmacéuticos activos, PhACs) en diferentes matrices y compartimentos ambientales como son las aguas superficiales y subterráneas, suelos, biota y parte comestible de plantas agrícolas (aus der Beek et al., 2016; de Santiago-Martín et al., 2020; Petrie et al., 2015; Wilkinson et al., 2017). Con respecto a los PhACs, se ha identificado que las principales fuentes de entrada al medioambiente son los efluentes de las estaciones de aguas residuales urbanas (EDARs) (agua depurada y fango estabilizado) debido al consumo humano y excreción en los hogares y hospitales (Boxall et al., 2012; Gogoi et al., 2018; Krzeminski et al., 2019; Verlicchi and Zambello, 2015).

Inicialmente, las EDARs convencionales no fueron diseñadas para la eliminación/remoción de estos compuestos farmacéuticos tanto en línea de aguas como en línea de fangos. En este sentido, las concentraciones de diferentes PhACs en las aguas tratadas y el fango digerido son frecuentemente más altas que las concentraciones máximas predichas para que no causen efecto toxico al medioambiente (PNEC) (Verlicchi et al., 2012; Verlicchi and Zambello, 2015). Además, las EDARs convencionales han demostrado cada vez más problemas para cumplir los límites de vertido más estrictos en términos de vertido de nutrientes, así como una mayor dificultad de reducir los costes de operación y aumentar la eficiencia de operación tanto en línea de aguas como en línea de fangos (COM(2019) 128, 2019; Güneş et al., 2019; Xu et al., 2020). En consecuencia, las EDARs antiguas necesitan ser reformadas y modernizadas con procesos avanzados de eliminación biológicas de nutrientes y de digestión de fangos, que a su vez puedan ser operadas para eliminar al máximo los diferentes micro contaminantes orgánicos como son los PhACs.

Con estos antecedentes, en la presente Tesis Doctoral se ha evaluado el balance y la eliminación de 27 PhACs tanto en línea de aguas como en línea de fangos en una planta piloto a escala que trata agua residual urbana de la EDAR Murcia Este. En línea de aguas se han comparado y estudiado un sistema convencional de eliminación de nutrientes A^2O operando a diferentes condiciones operacionales junto con un sistema avanzado A^2O-

IFAS, mientras que en línea de fango se ha estudiado un sistema avanzado de digestión bifásica. La investigación se ha planteado desde un punto de vista multidisciplinar, en busca de vínculos entre la eficacia de la remoción de los PhACs, el rendimiento de sistema de tratamiento, los parámetros de operación, y la estructura de las comunidades microbianas, tanto en la línea de aguas como en la línea de fangos de la planta piloto.

1. Parámetros operacionales y rendimientos en términos de materia orgánica y nutrientes en la línea de aguas: Sistema A^2O vs A^2O-IFAS

En las últimas décadas, los sistemas convencionales de eliminación de nutrientes biológica (BNR) han sido ampliamente estudiados y han llegado a obtener buenos rendimientos. Sin embargo, estos sistemas normalmente requieren operar con altos HRT (volúmenes de biorreactor grandes) y altas edades de fango (SRT), especialmente en climas fríos, donde los costes de operación e inversión son mayores (Ashrafi et al., 2019). Asimismo, la creciente disminución de los valores límites de vertido del efluente, la creciente reutilización del agua, la necesidad de usar menor área de implementación y la necesidad de mejorar las sobrecargadas EDARs, han exigido una continua investigación en procesos avanzados de eliminación de nutrientes y contaminantes emergentes. De entre las nuevas tecnologías, los reactores de biopelícula de lecho móvil (MBBR) y más concretamente la híbrida MBBR (IFAS) se postula a ser una tecnología prometedora debido a sus numerosas ventajas frente a los procesos convencionales (CAS) (di Biase et al., 2019; Leyva-Díaz et al., 2020). Una de las principales ventajas del sistema IFAS para ser aplicado ampliamente es la capacidad que tiene de transformar las antiguas EDARs de fango activo convencionales que se encuentran sobrecargadas sin necesidad de obra civil compleja y a un coste muy bajo al añadir un cierto porcentaje de relleno en los reactores biológicos (Leyva-Díaz et al., 2020).

Para demostrar las ventajas del sistema IFAS reportadas en la literatura, en la presente Tesis Doctoral se ha comparado el sistema IFAS y el CAS en una planta piloto de eliminación de nutrientes A^2O diseñada a escala de la EDAR Murcia Este. Para ello, la piloto ha operado en tres fases de experimentación diferenciadas; las dos primeras fases (Fase I y II) en sistema A^2O convencional con diferentes parámetros operacionales (principalmente MLSS y F/M ratio) y la última fase (Fase III) en sistema A^2O-IFAS. Para observar de una forma significativa las ventajas del sistema IFAS y simular una EDAR sobrecargada, la planta piloto fue operada al menor HRT posible (entre 7.0 y 6.6 horas) en vez de al HRT de diseño (10 horas).

Mejores rendimientos de eliminación de materia orgánica y nutrientes (nitrógeno y fósforo) se obtuvieron en la Fase II con respecto a la Fase I debido principalmente a que la Fase II operó con el doble de concentración de biomasa (MLSS), con menor F/M ratio, con mayor carga orgánica y con un mayor COD/TN ratio que favoreció la eliminación de nutrientes (Table 1, Chapter 1). Altos COD/TN ratios aumenta la carga orgánica asimilable para las bacterias heterótrofas desnitrificantes y los microorganismos acumuladores de fosforo (PAOs) encargados de la eliminación del nitrógeno y fósforo, respectivamente (Barnard and Abraham, 2006; Mannina et al., 2018; Tchobanoglous et al., 2003). Análogamente, menores concentraciones de materia orgánica y nutrientes en el efluente y mayores ratios de eliminación de nitrógeno y fósforo se produjeron en la Fase III con respecto a las Fases I y II (Table S1 y S2, Chapter 4) al operar como sistema A^2O-IFAS con la introducción de un 50% de relleno plástico AnoxKaldnes K5 en los compartimentos aeróbicos. La mejora de rendimiento del sistema A^2O-IFAS ocurre a pesar de que se opera con menor COD/TN ratio con respecto a la Fase II, con menor recirculación de fango activo secundario (RAS) y menor recirculación de fango activo (MLR), con menor concentración de biomasa suspensa (MLSS), con menor HRT, así como con una mayor carga de nitrógeno (NLR) y de fósforo (PLR) (Table S1 y S2, Chapter 4). Estos hechos ponen en valor la capacidad que tiene la biomasa adherida para mejorar la eliminación de nutrientes y materia orgánica (Barnard and Abraham, 2006; Mannina et al., 2018, 2017; Regmi et al., 2011). Conjuntamente, durante la investigación de la Fase III quedó demostrado que la eliminación de nitrógeno total fue similar durante toda la fase de experimentación, independientemente de la recirculación interna aplicada (MLR); con lo cual, un alto porcentaje de nitrógeno se elimina mediante el proceso de nitrificación-desnitrificación simultanea debido a la combinación de diferentes condiciones redox proporcionadas por la biopelícula y a la baja concentración de oxígeno disuelto de operación (0.5-1.0 mgL^{-1}) (Ashrafi et al., 2019; Iannacone et al., 2019).

Las condiciones de operación aplicadas a nuestro sistema A^2O-IFAS nos proporciona diferentes ventajas frente al A^2O convencional. Por ejemplo, los costes de operación y de inversión son menores debido a que se obtienen mejores rendimientos al opera con menor recirculación interna y externa (MLR and RAS), menor concentración de MLSS, así como menor HRT y menor edad de fango activo suspendido (ML-SRT) (Bashar et al., 2018; Güneş et al., 2019). Al mismo tiempo, cuando operamos a menor ML-SRT se produce un fango en exceso con alto porcentaje en volátiles (MLVSS de 84% frente a 79%, Table S1,

Chapter 4) que incrementa considerablemente la capacidad de biodegradación anaeróbica, así como la producción de biogás cuando el fango es estabilizado mediante digestión anaeróbica (Bolzonella et al., 2005; Gonzalez et al., 2018; Xu et al., 2020). Además, bajos ML-SRT (< 5 días) es preferible para los microrganismos de rápido crecimiento como son los PAOs y los heterótrofos desnitrificantes que son microorganismos encargados de la eliminación del fósforo y nitrógeno (Onnis-Hayden et al., 2011). Con respecto a la capacidad de sedimentación del fango activo IFAS, Kim et al. (2010) obtuvo peor sedimentación del fango activo en el sistema IFAS con respecto al CAS, pero la sedimentación fue aceptable. Esto concuerda con nuestra investigación, donde el proceso de sedimentación fue adecuado en el sistema IFAS, pero fue ligeramente inferior al sistema A^2O, ya que se obtuvo un índice volumétrico de fangos (SVI) mayor con respecto el sistema convencional en Fase I y similar con respecto a la Fase II. Contrariamente, Di Trapani et al. (2010) obtuvo un resultado diferente, ellos concluyeron que el sistema IFAS incrementó la capacidad de sedimentación del fango con respecto al sistema CAS.

Para finalizar, al igual que los numerosos estudios que comparan la tecnología IFAS con el sistema CAS en términos de eficiencia y rendimientos (Araujo Junior et al., 2013; Bashar et al., 2018; Di Trapani et al., 2013, 2010; Güneş et al., 2019; Kim et al., 2010) la presente investigación ha demostrado que el sistema A^2O-IFAS incrementa los rendimientos de eliminación de materia orgánica y nutrientes a menor coste operacional y de inversión con respecto al sistema convencional homólogo A^2O. Por consiguiente, este es un sistema simple y eficiente para actualizar y mejorar las EDARs existentes o para nuevos diseños de biorreactores (di Biase et al., 2019; Leyva-Díaz et al., 2017).

2. Parámetros operacionales y rendimientos de la digestión bifásica del fango producido por la línea de aguas

El tratamiento y gestión de los fangos producidos por la línea de aguas de las EDARs urbanas es un reto importante ya que representa sobre el 50% de los costes operacionales globales y sobre el 30-40% del coste capital de muchas de estas instalaciones (Appels et al., 2008; Demirer and Othman, 2008). Se ha estimado que la producción media de fangos en las EDARs municipales en 2017 dentro de la EU28 fue de 58.9 g DM $cap.^{-1}d^{-1}$, donde España representa el valor más alto con 160.7 gDM $cap.^{-1}d^{-1}$ (Ivanová et al., 2018). Hoy en día, la digestión anaeróbica convencional (AD) de alta carga es la configuración más utilizada en las EDARs municipales. Sin embargo, la poca biodegradabilidad que suele tener los fangos secundarios (WAS), junto con los altos volúmenes de digestión, problemas

de espumas y digestiones inestables han hecho que se hayan estudiados otros métodos y configuraciones diferentes, como son los métodos de pretratamientos de fangos (p.ej. tratamientos térmicos, alcalinos y mecánicos de rotura, sonificación, ozono, etc) que requieren altas cantidades de energía y son generalmente caros, así como la digestión bifásica que opera en dos digestores en serie y no requieren costes adicionales (Demirer and Othman, 2008).

En este sentido, la digestión bifásica ha sido desarrollada y propuesta para mejorar la eficiencia y solucionar los problemas de la AD convencional (Ghosh, 1987; Ghosh et al., 1995; Leite et al., 2016; Oles et al., 1997). La digestión bifásica se produce en dos digestores en serie que permite la selección y enriquecimiento de microorganismos diferentes en cada digestor, esto se debe a las diferentes ratios de crecimiento entre los microorganismos acidogénicos y las arqueas metanogénicas (Sidhu et al., 2013; Smith et al., 2017). Las principales ventajas de la digestión bifásica son que este sistema permite un mejor control del pH y soporta mejor los picos de alta carga orgánica, pueden operar a SRT más cortos, mejora la eliminación de materia orgánica volátil, y proporciona mayor estabilidad de las comunidades microbianas. De esta manera, varios autores demostraron que la digestión bifásica puede ser más rentable y operar con digestores más pequeños que la AD convencional (Bhattacharya et al., 1996; Bolzonella et al., 2012, 2003; Demirel and Yenigün, 2002; Demirer and Othman, 2008; Ghosh, 1987; Ghosh et al., 1995; Leite et al., 2016; Maspolim et al., 2015a; Oles et al., 1997; Smith et al., 2017; Watts et al., 2006).

A tenor de las ventajas de la digestión bifásica descritas anteriormente, el fango primario y secundario producido por la línea de aguas durante el desarrollo de las fases experimentales de esta Tesis Doctoral se trató mediante digestión bifásica. Para una óptima producción de biogás y eliminación de sólidos volátiles en digestión bifásica es necesario una separación efectiva entre etapas, en el cual es importante conocer el SRT óptimo del digestor acidogénico (AcD) (primera etapa) que produzca el máximo de ácidos grasos volátiles (VFA) necesarios para las arqueas productoras de metano en el digestor metanogénico secundario (MD) (segunda etapa) (Ghosh, 1987; Martín-Pascual et al., 2017; Maspolim et al., 2015a; Ponsá et al., 2008). Por consiguiente, la investigación se realizó con diferentes SRT in dos fases de experimentación; la Fase I operó con un SRT de 2 y 12 días para el AcD y MD respectivamente, y la Fase II operó con 5 y 24 días en el AcD y MD respectivamente.

Finalmente se determinó que la separación de los microorganismos (acidogénicos y metanogénicos) en los digestores solo fue efectiva en la Fase I, ya que en la Fase II se obtuvieron concentraciones de metano y producción de metano similares entre los dos digestores (AcD y MD) (Table 1, Chapter 3). Además, el pH fue alto y la ratio VFA/ALK muy baja comparado con los valores normales de un digestor acidogénico de digestión bifásica de lodos de depuradoras (Ghosh, 1987; Martín-Pascual et al., 2017; Maspolim et al., 2015a; Ponsá et al., 2008). Por consiguiente, nuestros resultados mostraron que para un fango espesado mixto que contiene un porcentaje de sólidos totales bajo (%TS<3, Table 1, Chapter 3) con respecto a los obtenidos normalmente en las EDARs (Appels et al., 2008; Ponsá et al., 2008; Tchobanoglous et al., 2003) es necesario un SRT inferior a 5 días para obtener una separación optima entre fases. Asimismo, Ponsá et al. (2008) obtuvo un SRT óptimo en el AcD termófilo de 4 días para un fango mixto con una concentración de sólidos alta (%TS>4) y de 1 día para un fango mixto con baja concentración de sólidos baja (%TS<3). Análogamente, Ghosh (1987) obtuvo los mejores rendimientos en una digestión bifásica mesofílica cuando operaba el AcD con SRT de 2 días y a pH de 6. Igualmente, Martín-Pascual et al. (2017) obtuvo la máxima producción de metano en el MD cuando operaba el AcD con 2.18 días, alcanzando así la máxima concentración de VFA. Sin embargo, Maspolim et al. (2015a) obtuvo la máxima producción de VFA con 5 días de SRT en el AcD en comparación con 2 y 3 días de SRT, aunque el pH de operación fue mantenido manualmente constante a 5.5 y el %TS fue de 3.2%.

Para finalizar, comentar que la Fase II obtuvo una mayor producción global absoluta de biogás y metano debido a que operó al doble de SRT (14 días en Fase I frente a 29 días en Fase II) (Table 1, Chapter 3). Sin embargo, la producción de metano y biogás por sólidos volátiles eliminado, así como la ratio de producción volumétrica fue mayor en el digestor metanogénico de la Fase I; este hecho se debe a que la digestión bifásica fue más eficaz en la Fase I al producirse una correcta separación entre los procesos de acidogénesis y metanogénesis en el AcD y MD respectivamente. Por consiguiente, según lo expuesto en bibliografía y lo analizado en este estudio, para un fango espesado de baja concentración de sólidos totales (%ST<3%) el SRT óptimo del AcD debe de ser inferior a 5 días y preferiblemente debe de estar entre 1 y 2 días, así como el óptimo SRT del MD debe de estar entre 12 y 24 días, en el cual el SRT óptimo debe de ser encontrado después de un balance de coste-beneficio global realizado al proceso.

3. Presencia y balance másico de los 27 PhACs seleccionados en línea de aguas y línea de fangos.

Durante las dos últimas décadas, se han realizado una gran cantidad de estudios en las EDARs de todo el mundo, a escala real y piloto, donde se estudiaron la presencia y la eficiencia de remoción de una gran variedad de PhACs. Estos estudios fueron realizados mayoritariamente en la línea de aguas de las EDARs o plantas piloto que operan con sistemas CAS y MBR, en el cual existen pocos estudios a escala real o piloto que analicen la presencia y eficacia de remoción en sistemas IFAS en línea de aguas y en sistemas de digestión anaeróbica bifásica en línea de fangos (Grandclément et al., 2017; Shreve and Brennan, 2019; Tran et al., 2018; Verlicchi et al., 2012; Verlicchi and Zambello, 2015).

En la presente Tesis Doctoral se ha estudiado la presencia, el balance másico y la eficiencia de eliminación de 27 PhACs (en línea de aguas y en línea de fangos) en tres fases de investigación en una planta piloto que trata el agua residual de la EDAR Murcia Este. Además, se ha realizado una revisión bibliográfica sobre la entrada y salida de los 27 PhACs seleccionados tanto en línea de aguas como en fango de las EDARs y plantas pilotos que tratan aguas residuales urbanas (consultar punto I.6).

Generalmente, existe una gran variabilidad de concentración en el influente y efluente entre los diferentes estudios revisados (Figure I-2 y Figure I-3). Asimismo, la mayoría de los PhACs fueron detectados en las tres Fases de operación con rangos de concentraciones muy amplios, dónde las concentraciones de influente de las Fases I y II fueron significativamente similares para la mayoría de los PhACs; sin embargo, la Fase III obtuvo concentraciones en el influente significativamente inferiores para 8 PhACs (Table S3, Chapter 4). En general, las concentraciones medias evaluadas en las tres fases de todos los PhACs entran dentro de los rangos encontrados en bibliografía. Sin embargo, las concentraciones medias evaluadas en el influente en las tres fases experimentales fueron mayoritariamente más bajas que las concentraciones medias calculadas mediante los datos de bibliografía (Figure I-2), excepto para el diclofenaco, naproxeno y gemfibrozil en Fase I, el acetaminofén en Fase I y II, el ketoprofeno en Fase I y III, así como el ofloxacino, la furosemida y el lorazepam para las tres Fases. Igualmente ocurre con las concentraciones del efluente secundario, en el cual, las concentraciones medias de los PhACs de cada fase de investigación fueron mayoritariamente más bajas que las medias calculadas mediante los datos de bibliografía (Figure I-3), excepto para el gemfibrozil en Fase I, los AIAPs

acetaminofén, diclofenaco, ibuprofeno y naproxeno en Fase I y II, así como los compuestos ketoprofeno, ofloxacino, furosemida, hidroclorotiazida y lorazepam en las tres Fases.

Para poder identificar los PhACs susceptibles de dañar el ecosistema acuático, se realizaron comparaciones entre el valor medio del efluente obtenido en las tres fases de operación y el obtenido de los datos bibliográficos con el valor límite inferior de concentración a partir del cual se observan efectos tóxicos (PNEC). El mínimo PNEC de los 27 PhACs seleccionados se muestra en la Table I-4 y fue obtenido de la bibliografía reciente (Orias and Perrodin, 2013; Tran et al., 2018; Verlicchi et al., 2012). Los compuestos que superen el PNEC en el efluente son susceptibles de dañar el ecosistema acuático si son vertidos sin tratamiento terciarios en zonas con factor de dilución bajos. En este sentido, las Fases I y II junto con los valores medios calculados de bibliografía obtuvieron el mismo número de PhACs (12) y los mismos compuestos (Table I-4 y Table S4, Chapter 4) a excepción del acetaminofén, lorazepam y bezafibrato que fue mayor en Fase I y II, así como el metoprolol, diazepam y furosemida que fue mayor en los datos bibliográficos. Sin embargo, debido a las menores concentraciones de entrada detectadas para 8 PhACs en Fase III (sistema IFAS) junto con los mayores índices de eliminación/remoción detectados en el sistema A^2O-IFAS (Table 2, Chapter 4) los compuestos susceptibles a dañar el medioambiente se redujeron a 7 en Fase III (Table S4, Capítulo 4).

Al igual que para la línea de aguas, en la línea de fango se ha realizado una revisión bibliográfica de la presencia de los 27 PhACs tanto a la entrada (fango primario, mixto o secundario) como a la salida del proceso de digestión anaeróbica (fango digerido) (Figure I-4 y Figure I-5). En general, el volumen de estudios y de datos encontrados es mucho menor que los encontrados para la línea de aguas, debido principalmente a la mayor complejidad de analizar bajas concentraciones de PhACs en la matriz de un fango (Verlicchi and Zambello, 2015). Sin embargo, es necesario conocer la cantidad de compuesto que pasa y se elimina en la línea de fangos para poder cerrar así el balance global (Ashfaq et al., 2017; Blair et al., 2015; Jelić et al., 2011; Park et al., 2017; Wang et al., 2018). Además, es importante conocer la concentración final del fango digerido y estabilizado (se han reportado concentraciones máximas de hasta 80 μg g^{-1}DM para ofloxacino y 8.1 μg g^{-1}DM para el ibuprofeno, Narumiya et al. 2013 y Phan et al. 2018 respectivamente) que puede ser aplicado en suelo agrícola, ya que aproximadamente el

40% del lodo que se produce en las EDARs de la EU es utilizado como enmienda orgánica en agricultura y sobre el 12% es utilizado para producir compost (Ivanová et al., 2018).

En general, las concentraciones medias del fango mixto espesado y del fango digerido evaluadas en las Fases I y II entran dentro del rango de los datos revisados en bibliografía (Table 2, Figure I-4 y I-5, Chapter 3), a excepción del regulador lipídico fenofibrato y el medicamento psiquiátrico paroxetina que fueron considerablemente más altas. Además, las concentraciones analizadas en el fango espesado fueron estadísticamente similares entre Fases I y II, a excepción de 7 PhACs (Table 2, Chapter 3). Es importante destacar que el antibiótico ofloxacino fue detectado a concentraciones considerablemente más altas en los tres tipos de fangos analizados con una alta variabilidad entre todas las muestras analizadas (rango entre 0.035 y 122 μg g^{-1} DM, Table S3, Chapter 3). Esta alta variabilidad ha sido detectada anteriormente por Narumiya et al. (2013) y Verlicchi and Zambello (2015). Este hecho refleja el uso de este medicamento para tratar brotes temporales de infecciones; asimismo, Coutu et al. (2013) encontraron una clara estacionalidad en el patrón de detección de concentraciones de ofloxacino en aguas residuales urbanas.

Para tener una idea de la cantidad de fármaco que entra en las EDARs y que salen mediante efluente secundario y fango digerido o estabilizado, se ha calculado la carga másica por cada 1000 habitantes (ML) que entra a la planta piloto en las Fases I y II, tanto en línea de aguas como en la de fangos (Table 4, Chapter 1; Table 3, Chapter 2; Table 3, Chapter 3). Además, se ha realizado una revisión bibliográfica (Figure I-6 y I-7) de la carga másica por cada 1000 habitantes correspondiente a la entrada y salida de la línea de aguas. En general, la carga másica de entrada calculada por cada PhAC en Fase I y II se ubica dentro del rango revisado en bibliografía (Figure I-6) a excepción de los AIAPs acetaminofén y ketoprofeno que fueron considerablemente más altos que los valores máximos encontrados en la bibliografía. No obstante, hay que tener en cuenta que la comparación entre estudios es complicada ya que los valores de ML están altamente influenciados por los diferentes patrones de consumo, la zona geográfica y el momento de muestreo (Jelić et al., 2011; Papageorgiou et al., 2016; Park et al., 2017; Pereira et al., 2015).

Resumidamente, se obtiene una carga másica de entrada total para los 27 PhACs de 46267 mg $1000h^{-1}$ d^{-1} en Fase I y 48172 mg $1000h^{-1}$ d^{-1} en Fase II; de las cuales, 553 mg $1000h^{-1}$ d^{-1} y 253 mg $1000h^{-1}$ d^{-1} pasa al fango mixto espesado en Fase I y II

respectivamente. Con lo cual, los porcentajes de remoción máximos por sorción en línea de aguas es de un 1.20% en Fase I y un 0.53% en Fase II. Estos porcentajes son máximos porque no se analiza la parte filtrada del agua de entrada, con lo cual, no se ha tenido en cuenta la masa de los compuestos sorbidos en los sólidos filtrables del agua de entrada. Por consiguiente, los valores de eliminación por sorción reales son iguales o menores a los calculados anteriormente. Por otro lado, la carga másica de salida por línea de aguas es de 7227 mg $1000h^{-1}$ d^{-1} para la Fase I y 17851 mg $1000h^{-1}$ d^{-1} para la Fase II (el valor tan alto de la Fase II se debe al compuesto recalcitrante ofloxacino que corresponde al 82 % del total en Fase II (Table 3, Chapter 2). Por consiguiente, el porcentaje de biodegradación global másico fue de 84.4% para Fase I y 62.9% para Fase II. El porcentaje de biodegradación global másico más bajo de la Fase II se debe a la proporción másica tan alta del compuesto no biodegradable ofloxacino (82% del total) en la Fase II; por lo tanto, esto no significa que la Fase I fue más eficiente que la Fase II, ya que los índices de eliminación/remoción individuales para la mayoría de los PhAC fueron mayores en Fase II que en Fase I (Se discute en el siguiente punto).

Según el balance másico global realizado a los 27 PhACs seleccionados, la eliminación/remoción se debe principalmente al proceso de biodegradación, ya que el porcentaje de remoción global por sorción es inferior a 1.2% en las dos Fases. En general, muchos autores concluyeron mediante un cálculo completo del balance de masa (fase acuosa y suspendida) que la eliminación de la mayoría de los PhACs se atribuye principalmente al proceso de biodegradación/biotransformación en el tratamiento biológico secundario (Ashfaq et al., 2017; Gao et al., 2012c; Jelić et al., 2012; Verlicchi et al., 2012; Y. Wang et al., 2018; Xue et al., 2010; Yan et al., 2014b; Zorita et al., 2009). Sin embargo, otros autores destacan la importancia de la eliminación por la sorción para varios PhAC como son algunos antibióticos y fluoroquinolonas (Guerra et al., 2014; Jia et al., 2012; Martínez-Alcalá et al., 2017; Petrie et al., 2014; Yan et al., 2014b), así como fenofibrato, diazepam, claritromicina e hidroclorotiazida (Jelić et al., 2011). En este sentido, la remoción mediante sorción en la línea de agua podría ser una vía de remoción significativa para compuestos con alta hidrofobicidad representada por el coeficiente octanol-agua (K_{ow}) y más específico por el potencial de sorción indicado por el coeficiente de sorción experimental sólido-agua (K_d) (Table I-3). Hasta la fecha, se ha aceptado ampliamente una regla simple, si el compuesto tiene un alto potencial de sorción (K_d>500 L Kg^{-1} o log K_{ow}> 2.5), los PhAC tienden a sorberse en la materia orgánica y partículas,

siendo un candidato para ser eliminado por los fangos primarios y secundarios (RE> 20%) (Luo et al., 2014; Ternes y Joss, 2015; Tran et al., 2018; Verlicchi et al., 2012). En este sentido, si descartamos los compuestos que se detectaron con concentraciones <10 ng g^{-1} y una frecuencia <50% en línea de fangos, únicamente la paroxetina y el fenofibrato en las Fases I y II, así como los fármacos ofloxacino y sotalol en la Fase I y la codeína en Fase II fueron removidos mediante sorción en más de un 5%. Este hecho se debe a su alta hidrofobicidad reflejado en los altos valores de K_{ow} y K_d (Table I-3). Destacar que la paroxetina y el fenofibrato obtuvieron altas cargas másicas en el fango espesado (Table 3, Capítulo 3) a pesar de que no fue detectado en las muestras filtradas del influente (Table 1, Capítulo 2).

4. Eficiencia de eliminación/remoción (RE) de los PhACs y relaciones con los parámetros operacionales en línea de aguas: Sistema A^2O-IFAS versus sistema A^2O

El término eliminación/remoción (RE) de PhACs en la línea de aguas se refiere a la pérdida del compuesto original durante el proceso de depuración, pérdida que se debe a diferentes procesos physico-químicos y mecanismos biológicos (sorción a la materia sólida, volatilización y biodegradación/biotransformación). La remoción mediante volatilización es considerada residual debida al bajo valor de la constante de vapor de solubilidad de Henry (H) para los PhACs seleccionados (Table I-3). La remoción de los PhACs mediante sorción en la línea de aguas comienza inicialmente con los procesos de pretratamiento y la decantación primaria y finaliza con la decantación secundaria después del reactor biológico.

Numerosos estudios han determinado que la remoción en los procesos de pretratamiento y decantación primaria es muy pequeña o residual (Ashfaq et al., 2017; Carballa et al., 2005; Verlicchi et al., 2012; Watkinson et al., 2007; Yan et al., 2014), incluso el compuesto original ha sido a veces incrementado debido a los procesos de desorción de la parte sólida o a la desconjunción/transformación de los metabolitos en el compuesto original (Ashfaq et al., 2017; Carballa et al., 2004; Göbel et al., 2005; Zorita et al., 2009). Sin embargo, existen compuestos altamente hidrofóbicos que se encuentran mayoritariamente adsorbido/absorbido en las partículas sólidas sedimentables del agua residual y que son removidos en gran medida mediante la decantación primaria, tal y como ocurre con el fenofibrato y la paroxetina en nuestro estudio. En este sentido, Petrie et al. (2014) obtuvieron eficiencias de remoción de entre 24 y 55% para naproxeno,

acetaminofeno e ibuprofeno en la fase acuosa durante la sedimentación primaria. Del mismo modo, Zorita et al. (2009) obtuvieron una remoción en la sedimentación primaria de17, 22 y 57% para ibuprofeno, naproxeno y diclofenaco respectivamente. Asimismo, un 13% de remoción obtuvo Stasinakis et al. (2013) para el diclofenaco en la decantación primaria.

Posteriormente, si el compuesto no es removido en la decantación primaria este puede ser removido igualmente en la decantación secundaria, donde existe la posibilidad de que ciertos fármacos con alta hidrofobicidad (altos K_d y K_{ow}) sean eliminados en gran medida mediante sorción al fango activo en exceso. No obstante, tal y como se ha discutido en el apartado anterior, la mayoría de autores mencionados anteriormente que han realizado un balance másico completo, han concluido que el proceso de biodegradación/biotransformación producido en el biorreactor es el causante principal de la remoción de la mayoría de los PhACs en el tratamiento secundario. En este sentido, la mayoría de los PhACs seleccionados que han obtenido una concentración >20 ng L^{-1} y frecuencia de determinación >50% en la parte acuosa del influente de las Fases I y II, fueron removidos por sorción como máximo en un porcentaje inferior al 5% en las Fases de estudio I y II, excepto los fármacos ofloxacino y sotalol en Fase I (38 y 37% respectivamente) y la codeína (14%) en Fase II.

Finalmente, durante el paso por el tratamiento biológico secundario (reactor biológico), diferentes microorganismos del fango activo pueden biodegradar los compuestos microorgánicos (p.ej. PhACs) mediante mecanismos anabólicos o cometabólicos. Según varios autores, la biodegradación de PhACs es realizada principalmente por mecanismos cometabólicos debido a la baja concentración presente de estos compuestos en las aguas residuales (Fernandez-Fontaina et al., 2012; Fischer and Majewsky, 2014; Grandclément et al., 2017; Tran et al., 2013). Varios estudios han señalado que los procesos de biodegradación/biotransformación de los PhACs están altamente relacionados con la composición y características de la biomasa, que a su vez está relacionada con la configuración y los parámetros operacionales/ambientales del proceso de depuración (p.ej. MLSS, SRT, HRT, F/M ratio, temperatura, entrada de BOD_5, COD, TN y TP) así como con el rendimiento de depuración (p.ej. ratio de nitrificación, ORR, NRR y PRR) (De La Torre et al., 2015; Fernandez-Fontaina et al., 2012; Grandclément et al., 2017; Guerra et al., 2014; Maeng et al., 2013; Tran et al., 2013; Verlicchi et al., 2012; Xia et al., 2012). Debido a ello, durante la revisión bibliográfica se

ha encontrado una gran variabilidad de RE por cada PhAC entre los diferentes estudios revisados, principalmente entre los sistemas CAS (Figure I-11) y en menor medida entre los sistemas MBR (Figure I-12) e IFAS (Figure I-13). De igual forma, también se encontraron diferencias significativas de RE por cada PhAC entre las diferentes tecnologías (CAS, MBR e IFAS) (Table I-9). En este sentido, en la presente Tesis Doctoral se han encontrado también diferencias significativas de RE para 7 PhACs entre las Fases I y II que operan con diferentes parámetros operacionales, pero con el mismo sistema A^2O de tratamiento (Table 5, Chapter 1; Table 4, Chapter 2), así como diferencias significativas para 11 PhACs entre dos sistemas diferentes; A^2O-IFAS aplicado en la Fase III y el sistema A^2O convencional aplicado en Fases I y II.

Los PhACs que presentaron concentraciones medias <20 ng L^{-1} y frecuencia de detención <50% (propifenazona, sulfametoxazol, sulfametazina, sulfadiazina, metoprolol, fenofibrato, diazepam y paroxetina) no se han tenido en cuenta en el cálculo de los RE. De los 19 PhACs restantes, los PhACs ibuprofeno, naproxeno, claritromicina, gemfibrozil y bezafibrato obtuvieron RE significativamente más altos en la Fase II comparados con la Fase I, mientras que el sotalol y la hidroclorotiazida obtuvieron RE bajas pero significativamente más altos en la Fase I que en la Fase II (Table 5, Chapter 1; Table 4, Chapter 2). Las RE significativamente más altos en la Fase II fueron correlacionados principalmente con la mayor concentración de biomasa (MLSS) y el menor F/M ratio de operación aplicado en Fase II, además de una mayor carga de entrada de NH_4^+ y una mayor eliminación de nitrógeno total (NRR) (Figure 2, Chapter 1; Table S6, Chapter 2). Altos MLSS y bajos F/M ratio en los biorreactores pueden incrementar la biodegradación/biotransformación de los PhACs más biodegradables (Grandclément et al., 2017; Verlicchi et al., 2012; Weiss and Reemtsma, 2008). Además, altas cargas de N-NH_4^+ en los biorreactores que operan con altos SRT (>12 días) producen altos ratios de nitrificación; se ha determinado que una alta actividad de las bacterias nitrificantes está asociado a una mayor capacidad de biodegradación de una gran variedad de contaminantes microorgánicos a través de reacciones cometabólicas mediadas por las enzimas amonio monooxigenasa (Fernandez-Fontaina et al., 2012; Guerra et al., 2014; Kumwimba et al., 2019). Además, altos índices de eliminación de nitrógeno total (NRR) han sido relacionados también con una alta capacidad de eliminación de PhACs (Helbling et al., 2012; Thiebault et al., 2017).

Durante las últimas décadas, se han realizado varios estudios que han comparado directamente las RE de varios PhACs entre los sistemas CAS o MBR con los sistemas MBBR o IFAS (De La Torre et al., 2015; Shreve and Brennan, 2019). Por ejemplo, De La Torre et al. (2015), Falås et al. (2012) y Luo et al. (2015) encontraron mejores RE de los PhACs ketoprofeno y gemfibrozil en el sistema IFAS comparado con CAS y MBR, destacando la importancia de operar a altas concentraciones MLSS y altos SRT que dan una relación F/M ratio baja (De La Torre et al., 2015). Asimismo, Murray et al. (2017) obtuvo en biorreactores a escala de laboratorio alimentados con aguas residuales urbanas reales, RE más altas para el atenolol y la trimetoprima en el sistema IFAS comparado con el sistema CAS de control. Igualmente, Falås et al. (2013), (2012) y Jewell et al. (2016) demostraron en biorreactores a escala laboratorio que la biomasa adherida contribuyó significativamente a la eliminación de algunos PhAC en los procesos IFAS comparados con la biomasa suspendida.

A pesar de estas investigaciones, se necesita un mayor número de estudios que operen a escala real o piloto y que comparen directamente los sistemas IFAS con los sistemas CAS o MBR en relación a la remoción de PhACs (Grandclément et al., 2017; Shreve and Brennan, 2019). En este sentido, en la presente Tesis Doctoral se ha comparado el sistema A^2O-IFAS (Fase III) con el sistema convencional A^2O (Fase I y II). De los 19 PhACs que pudieron ser analizados en función de su RE, la mayoría (11 PhACs) presentan valores de RE medios más altos en Fase III que en las Fases I y II; de los cuales, el número de PhACs con diferencias significativas más altas fue mayor entre las Fases III y I (8) que entre la Fases III y II (5) (Table S5, Chapter 4); en el cual, solo el diurético hidroclorotiazida fue significativamente más bajo en Fase III comparado con la Fase I (-12 vs 33 %). Los parámetros operacionales que más influyeron en la mejora significativa de las RE entre fases fueron principalmente la mayor concentración de biomasa (MLSS) y el menor valor del F/M ratio para los PhACs ibuprofeno, naproxeno, ketoprofeno, gemfibrozil, atenolol, claritromicina y bezafibrato (Figure 2, Chapter 1; Table S6, Chapters 2 y 4). Asimismo, altas cargas de NH_4^+, junto con altos rendimientos de eliminación de nitrógeno, materia orgánica y fósforo aumentaron las RE del naproxeno, claritromicina y bezafibrato (Table S6, Chapters 2 y 4). Es importante destacar que el sistema IFAS obtiene significativamente mejores RE para 5 PhACs (ibuprofeno, naproxeno, trimetoprima, sotalol y diclofenaco) con respecto a la Fase II, a pesar de que el sistema IFAS opera a menor concentración de biomasa global (MLSS +BFSS), menor HRT y mayor F/M ratio global que la Fase II (Table

2, Chapter 4). Este hecho pone en valor la capacidad que tiene el sistema IFAS para incrementar los RE de los compuestos más biodegradables como son el ibuprofeno y el naproxeno, así como los más recalcitrantes como son la trimetoprima, sotalol y diclofenaco. Esta mejor capacidad se debe a que la biomasa adherida crea diferentes condiciones redox en la biopelícula que mejora las RE de ciertos PhACs (Grandclément et al., 2017; Torresi et al., 2016). Además, la combinación de la biomasa suspendida y biomasa adherida a los soportes plásticos en el biorreactor A^2O-IFAS da una combinación de microorganismos de crecimiento lento (biomasa adherida) y microorganismos de crecimiento rápido (biomasa suspendida) que aumenta el SRT global y el rango de posibles cepas activas capaces de mejorar la tasa de biodegradación/biotransformación de varios PhAC y microcontaminantes orgánicos (Biswas and Turner, 2012; Escolà Casas and Bester, 2015; Falås et al., 2013; Grandclément et al., 2017; Leyva-Díaz et al., 2020; Ooi et al., 2018; Polesel et al., 2017; Shreve and Brennan, 2019). Finalmente, debido al costo asequible y la alta eficiencia demostrada en la eliminación de materia orgánica, nutrientes y microcontaminantes orgánicos, los sistemas IFAS se han convertido en una de las tecnologías más prometedoras para la mejora de las plantas de tratamiento de aguas residuales convencionales.

5. Eficiencia de eliminación (RE) de los PhACs y relaciones con los parámetros operacionales de la digestión bifásica en línea de fangos

La eficiencia de eliminación (RE) calculada en la línea de fangos se debe únicamente a la pérdida de compuesto original durante el proceso de digestión mediante biodegradación/biotransformación ya que durante el proceso de digestión y de análisis de las muestras no existe ningún proceso de separación que pueda remover los compuestos originales. Actualmente, existen bastante menos estudios sobre la RE de PhACs en línea de fangos que en línea de aguas. En este sentido, solo se han revisado un total de 16 estudios, donde 5 reportan datos sobre la RE de PhACs en digestores de aguas residuales a escala real y 11 reportaron datos de digestores diseñados a escala laboratorio y piloto donde probaron diferentes SRT y temperatura (mesofílico y termofílico) de operación. En el cual, solo hemos encontrado un trabajo que estudie las RE de PhACs en digestores de digestión bifásica y monofásica (Figure I-14).

Los rangos de RE de cada PhACs entre estudios no son tan elevados en línea de fangos como los reportados en línea de aguas, a excepción de los AIAPs ibuprofeno y diclofenaco. Es interesante destacar que las RE más altas (>80%) obtenidos para estos

AIAPs en digestión anaeróbica (AD) fueron añadidos en el influente para operar con concentraciones suficientemente altas (Carballa et al., 2007; Samaras et al., 2014, 2013; Zhou et al., 2017). Sin embargo, RE bajos y negativos se obtuvieron en estudios que no se añadieron al influente en los procesos de AD (Phan et al., 2018; Radjenović et al., 2009; Yang et al., 2017, 2016). En este sentido, Gonzalez-Gil et al. (2016) y Phan et al. (2018) señalaron que estas grandes diferencias de RE entre algunos PhACs pueden ser debido a que unos estudios utilizan compuestos añadidos y otros no. Asimismo, Dictor et al. (2003) comprobó que los PhACs añadidos nuevos son más fácilmente asimilables y biodegradables que los PhACs viejos ya presentes.

En las Fases I y II de experimentación, los PhACs acetaminofén, naproxeno, claritromicina, sulfametoxazol, trimetoprima e hidroclorotiazida obtuvieron en la digestión bifásica global REs medias altas (>80%) y porcentajes de variación estándar muy bajos (<10%) (Table 4, Chapter 3) independientemente de las condiciones operacionales, al igual que ocurre con los valores obtenidos en bibliografía para estos PhACs (Figure I-14); excepto para la hidroclorotiazida, donde varios autores observaron valores más bajos (Jelić et al., 2012; Radjenović et al., 2009). Asimismo, los PhACs que obtuvieron RE medios bajos (<30%) o negativos en las Fases I y II (diclofenaco, ibuprofeno, gemfibrozil, carbamazepina, diazepam y paroxetina) fueron similares a los reportados en bibliografía (Figure I-14). Es importante resaltar que algunos compuestos que fueron fácilmente biodegradables en línea de aguas (condiciones aeróbicas principalmente, pero también anaeróbicas y anóxicas) como son el gemfibrozil y el ibuprofeno (Figure I-11; Table S5, Chapter 4) no han sido biodegradados en digestión anaeróbica (condiciones anaeróbicas estrictas), así como PhACs recalcitrantes en líneas de aguas como son el sulfametoxazol, la trimetoprima y la hidroclorotiazida (Figure I-11; Table S5, Chapter 4) han podido ser biodegradados fácilmente en digestión anaeróbica. Curiosamente, los antibióticos trimetoprima y sulfametoxazol fueron mejor biodegradados en el sistema A^2O-IFAS en línea de aguas (69 y 63%) debido a las mayores condiciones anaeróbicas generadas por la biopelícula en el reactor (Biswas and Turner, 2012; Torresi et al., 2016).

Muchos autores han comparado las REs de los PhACs en diferentes digestores que operan a diferentes temperaturas (rango mesófilo y termófilo), diferentes SRT y OLR. En general, la mayoría de los estudios (Carballa et al., 2007b, 2007a, 2006; Gonzalez-Gil et al., 2016; Malmborg y Magnér, 2015; Yang et al., 2017, 2016) no encontraron una correlación significativa entre los REs y los parámetros operativos (T, SRT y OLR).

Únicamente Carballa et al. (2006) observaron relaciones importantes entre la temperatura, el SRT y procesos de pretratamiento en la eliminación del ibuprofeno, y Zhou et al. (2017) demostró la influencia de altos SRT en la eliminación de la cafeína, el diclofenaco y el triclosán. En este sentido, Yang et al. (2016) postuló que esta aparente independencia entre los parámetros operacionales y los REs en AD podría estar relacionada con la variación de las concentraciones de entrada, con altos SRT y con que los PhACs no son el principal sustrato de los microorganismos anaeróbicos. Sin embargo, entre las dos fases experimentales y los dos tipos de reactores estudiados (AcD y MD) en la presente Tesis Doctoral, se obtuvieron diferencias significativas para 5 PhACs (Table 4, Chapter 3) entre los digestores (AcD y MD) de la Fase II que operaron con condiciones operacionales totalmente diferentes (Table 1, Capítulo 3), así como diferencias significativas para 7 PhACs (Table 4, Chapter 3) entre las Fases I y II para el digestor metanogénicos (MD) que operó con 12 días de SRT en Fase I y 24 días en Fase II; dónde, los altos REs obtenidos en Fase II para los PhACs codeína, ibuprofeno, claritromicina, propanolol, gemfibrozil, carbamazepina y lorazepam fueron correlacionados positivamente con el SRT (Table S4, Capítulo 3) a excepción de la codeína y la claritromicina.

6. Estructura y composición de bacterias, arqueas y hongos en el sistema A^2O de la línea de aguas

Las bacterias y los hongos heterótrofos son los microorganismos principales de la biodegradación/biotransformación de los compuestos orgánicos y nutrientes presentes en las aguas residuales urbanas. Al mismo tiempo, las arqueas tienen un rol importante en las relaciones metabólicas sintróficas encargadas de completar la mineralización de compuestos orgánicos, principalmente en las condiciones anaeróbicas del proceso A^2O (Nobu et al., 2015). Hoy en día, con las técnicas de cuantificación qPCR y las técnicas de secuenciación masiva en paralelo se ha podido cuantificar y caracterizar la comunidad microbiana de los procesos de tratamiento de aguas residuales y lodos de depuradora de una forma más profunda, barata y rápida que con las anteriores técnicas dependientes de cultivos (Ferrera and Sánchez, 2016; Vanwonterghem et al., 2014; Xia et al., 2018). En este sentido, en las dos primeras fases de investigación (Fase I y II) de esta Tesis Doctoral se han cuantificado mediante qPCR el número de copias de genes marcadores de bacterias totales, arqueas totales, micolatas y hongos en las muestras de fango activo del biorreactor A^2O. Además, se estudió la diversidad de bacterias, arqueas y hongos mediante secuenciación masiva en paralelo utilizando la plataforma Illumina.

La abundancia absoluta de bacterias totales por litro de fango activo fue de un orden de magnitud mayor en la Fase II que en la Fase I (Table S5A, Chapter 1). Por el contrario, la abundancia de arqueas fue significativamente mayor en la Fase I que en la Fase II. El grupo Mycolata solo se detectó durante la Fase II y el número de copias de ARNr 16S por L de fango activado se mantuvo en el orden de 10^{10}, mientras que las poblaciones de hongos solo se detectaron en la Fase I (Table S5A, Chapter 1). Sorprendentemente, Mycolata representó una alta proporción de las poblaciones bacterianas totales en el fango activo de la Fase II (promedio 31.02%). La mayor abundancia relativa se detectó el 14/12/2016, cuando el sistema operó con el índice volumétrico de fangos más alto (SVI, 137 mg L^{-1}) (Table 1, Chapter 1). En este sentido, varios autores obtuvieron correlaciones positivas importantes entre el SVI y la abundancia relativa de Mycolata (Griffin and Wells, 2017; Martins et al., 2004). Finalmente, en cuanto a la relación bacterias totales/arqueas totales, se detectaron diferencias significativas entre las dos fases (Figure 3B, Chapter 1). Según la literatura, las bacterias suelen ser más abundantes que las arqueas en los sistemas CAS, y las relaciones bacterias/arqueas varían de 1 a 10^5 (González-Martínez et al., 2017; Castellano-Hinojosa et al., 2018b). Los resultados del presente estudio sugieren que en las condiciones experimentales de la Fase II las bacterias proliferaron mejor frente a las arqueas. Igualmente, se han observado anteriormente interacciones negativas entre bacterias y arqueas en ecosistemas complejos (Steele et al., 2011)

La caracterización de la comunidad microbiana mediante secuenciación masiva en paralelo mostró que los filos bacterianos más abundantes fueron *Proteobacteria, Bacteroidetes, Chloroflexi* y *Firmicutes*. En particular, las proteobacterias dominaron en todas las muestras de ambas fases experimentales (Figure 1A, Chapter 2). A nivel de orden, las más abundantes fueron *Burkholderiales, Cytophagales, Caldilineales y Xanthomonadales* y a nivel de familia fueron *Comamonadaceae, Cytophagaceae, Caldilineaceae y Xanthomonadaceae*. La disimilitud observada entre fases fue del 47.93% mientras que la similitud de las muestras en cada fase fue de 61.48% en Fase I y 71.09% en Fase II (Table S8, Chapter 2).

Los filos *Proteobacterias, Bacteroidetes* y *Chloroflexi* han sido descritos como los grupos bacterianos predominantes en los fangos aeróbicos activados de las EDARs, ya sea en base a tecnologías convencionales o avanzadas (revisado por Ferrera y Sánchez, 2016; Xia et al., 2018). De entre estos filos, miembros del filo *Proteobacteria* son los más frecuentes y abundantes, particularmente en las EDARs urbanas (Cydzik-Kwiatkowska

and Zielińska, 2016). Entre estos, los miembros de la familia *Comamonadaceae* se consideran ubicuos en los fangos activos, donde cumplen una gran variedad de funciones como son la reducción de nitratos (Sadaie et al., 2007), la eliminación biológica de fósforo (Ge et al., 2015) y la biodegradación de sustratos complejos (Khan and Ongerth, 2002).

En cuanto a la diversidad de arqueas, se observó una importante abundancia de arqueas metanógenas, a pesar de que las muestras de fango se tomaron en la cámara aeróbica del biorreactor. Lo cual es habitual en los reactores biológicos de la línea de aguas de las EDARs operadas con aireación (Ferrera and Sánchez, 2016). Los géneros más abundantes fueron tanto metanógenas hidrogenotrofas (*Methanobrevibacter Methanobacterium, Methanolinea* y *Methanospirillum*) como acetotrofas (*Methanosaeta*) (Figure 1B, Chapter 2). Asimismo, se detectó el género *Nitrososphaera*, arquea oxidadoras de amonio (AOA), en una abundancia relativa significativa (de hasta el 15% en algunas muestras) (Figure 1B.3, Chapter 2).

Methanobacteriales, Methanomicrobiales y *Methanosarcinales* se detectan comúnmente en tratamientos de aguas residuales urbanas basados en diferentes tecnologías, incluidos los sistemas CAS (Ferrera y Sánchez, 2016). La configuración A^2O se caracteriza por zonas anaeróbicas, anóxicas y aeróbicas en serie, sometidas a recirculación interna y externa. En tales condiciones, generalmente no se encuentran diferencias significativas de la composición de las comunidades microbianas entre las cámaras con diferentes condiciones redox (Gómez-Silván et al., 2014; Kim et al., 2013; Phan et al., 2016). Asimismo, las arqueas metanogénicas presentes en las zonas anaeróbicas en condiciones óptimas para desarrollar su metabolismo ingresan a las zonas aireadas por recirculación, donde su contribución a la remoción de carbono y nitrógeno es controvertida, aunque algunos estudios apoyan su papel como potenciadores de algunas actividades biológicas a través de sus relaciones simbióticas con las bacterias (Ferrera y Sánchez, 2016).

De todas las muestras analizadas en las Fase I y II, los hongos estuvieron presente únicamente bajo las condiciones operacionales de la Fase I. Los resultaos revelaron una mayor diversidad y abundancia relativa de *Ascomycota* en comparación con *Basidiomycota* en el sistema A^2O de la Fase I, y en la mayoría de las muestras, los filotipos estrechamente relacionados con los *Saccharomycetes* mostraron las mayores abundancias relativas dentro de la comunidad fúngica (Figura 1C, Chapter 2), de acuerdo con el conocimiento de la diversidad de hongos en EDARs (Maza-Márquez et al., 2016b). *Ascomycota* es un grupo

de hongos extremadamente diverso, que representa >65% de estos microorganismos eucariotas en ambientes terrestres y acuáticos (Harms et al., 2011). Se han descrito varias especies capaces de biotransformar PhAC, incluidos AIAP, antibióticos o betabloqueantes (Olicón-Hernández et al., 2017).

7. Relaciones entre las variables ambientales/operacionales con la abundancia total y relativa de las bacterias, arqueas y hongos del sistema A^2O de la línea de aguas

En las últimas décadas se han obtenido importantes relaciones entre la abundancia y composición de la comunidad microbiana, las variables operacionales, así como los parámetros físico-químicos del influente de los biorreactores de las EDARs. Por consiguiente, numerosos estudios han indicado que la configuración del proceso, los parámetros operativos/ambientales y la composición físico-química del influente son los principales factores que impulsan el cambio de la estructura microbiana en las EDARs (Ferrera and Sánchez, 2016; Maza-Márquez et al., 2015; Muñoz-Palazon et al., 2018; Reboleiro-Rivas et al., 2016). En este sentido, los datos de abundancia absoluta de copias por litro del gen marcador para bacterias totales, arqueas totales, Mycolata y hongos, así como los datos de abundancia relativa de las poblaciones de bacterias y arqueas obtenidas mediante secuenciación (Illumina-sequencing) fueron ordenados mediante un análisis de escalado multidimensional no métrico (MDS) junto con los datos de los parámetros operacionales y físico-químicos más relevantes del sistema (Figure 4, Chapter 1; Figure 2, Chapter 2).

Los resultados presentados por la Figure 4 y Table S6 (Chapter 1) indicaron que las arqueas totales y los hongos se correlacionaron negativamente con la abundancia de Mycolata; en el cual, la F/M ratio y la temperatura (OT) se correlacionaron positivamente con las arqueas totales y hongos, mientras que la concentración de MLSS, COD y $N\text{-}NH_4^+$ fueron positivamente correlacionados con Mycolata. Estos resultados nos indican que las condiciones físico-químicas del influente y las condiciones operacionales del biorreactor ejercen influencias significativas entre la abundancia de los diferentes grupos microbianos principales, tal y como observaron estudios anteriormente realizados en EDARs urbanas (Griffin and Wells, 2017; Maza-Márquez et al., 2016a; Reboleiro-Rivas et al., 2015; Weithmann et al., 2016). El grupo denominado Mycolata está formado por bacterias filamentosas del filo *Actinobacteria* que se encuentran con frecuencia en los fangos activos de EDARs convencionales o avanzadas (Fang et al., 2013; Guo and Zhang, 2012; Maza-Márquez et al., 2016a, 2015). Estas son bacterias Gram positivas que contiene miembros

de los géneros *Nocardia*, *Rhodococcus*, *Skermania*, *Gordonia*, *Microthrix* y *Tsukamurella*, que pueden producir episodios de flotabilidad de fangos y espumas (bulking y foaming, respectivamente) si proliferan descontroladamente. Curiosamente, este grupo fue únicamente detectado por qPCR durante la Fase II, a pesar de que estas bacterias proliferan en una amplia gama de condiciones operacionales en las EDARs (de los Reyes, 2010). De acuerdo al análisis BIO-ENV, las variables operacionales OT, F/M ratio, carga orgánica y MLSS tuvieron una fuerte influencia en la abundancia de Mycolata en el sistema A^2O (Tabla S6, Chapter 1). Estas mismas relaciones fueron encontradas también en estudios anteriores realizados en EDARs urbanas (Asvapathanagul et al., 2012; Maza-Márquez et al., 2015; Parada-Albarracín et al., 2012). A pesar de la proliferación de estas bacterias filamentosas en Fase II, no se produjeron episodios de foaming o bulking. En este sentido, varios autores observaron también altas densidades de Mycolata en ausencia de foaming/bulking en un reactor MBR (Parada-Albarracín et al., 2012). En este sentido, dado que Mycolata es un grupo heterogéneo con una amplia diversidad filogenética y fisiológica, las cepas varían mucho en su capacidad para producir episodios de foaming o bulking (Petrovski et al., 2011).

Con respecto a la abundancia relativa de las bacterias y arqueas, los resultados mostrados por la Figure 2 y Table S11 (Chapter 2) indicaron dos tendencias muy claras en concordancia con las diferentes condiciones operacionales de cada fase (mayor OT y F/M ratio en la Fase I, así como mayor concentración de COD y BOD_5 en influente, mayor MLSS y SVI en Fase II), y con el incremento de eliminación de materia orgánica y nutrientes (ORR, NRR y PRR) registrados en Fase II. Del mismo modo que los resultados discutidos anteriormente, se puede observar el cambio de la estructura de la comunidad microbiana al cambiar las condiciones operacionales del sistema A^2O, tal y como se ha observado en otros estudios similares (Ju and Zhang, 2015; Kim et al., 2013; Reboleiro-Rivas et al., 2016).

Asimismo, la Figure 2A y la Table S11 (Chapter 2) mostraron correlaciones positivas con la abundancia relativa de varias familias de bacterias con las concentraciones de MLSS en el biorreactor y las concentraciones de COD, BOD_5 y $N\text{-}NH_4^+$ en el influente. Contrariamente, tendencias opuestas mostraron estas variables con la abundancia relativa de otras familias de bacterias. Algunas de las respuestas observadas en los grupos bacterianos a los cambios de los parámetros operacionales entre Fase I y II coincidieron con las observadas en estudios previos. El aumento de MLSS en la Fase II contribuyó a

una mayor abundancia relativa de poblaciones de *Caldilineaceae* y *Chloroflexi* (Figure 2A y Table S11A, Chapter 2), de acuerdo con las observaciones anteriores en un biorreactor de membrana de lecho móvil (MBMBR) que trata aguas residuales urbanas (Reboleiro-Rivas et al., 2016). Igualmente, Muszyński et al. (2015) analizaron la diversidad bacteriana en un sistema A^2O mediante la técnica FISH, donde observaron correlaciones positivas de las abundancias relativas de *Alfa-* y *Betaproteobacterias* con la concentración en el influente de DQO y TN, así como una correlación negativa con la OT, mientras que *Bacteroidetes* de *Saprospiraceae* siguió la tendencia opuesta.

En cuanto a las abundancias relativas de poblaciones de arqueas a nivel de género, el análisis BIO-ENV detectó que la concentración de $N\text{-}NH_4^+$ en los lodos activados fue el parámetro que mejor explicó la ordenación de las muestras (46,5%). Estos resultados indican un efecto importante de la concentración de $N\text{-}NH_4^+$ sobre la sucesión de metanógenos hidrogenotróficos y metilotróficos/acetoclásticos. La concentración de amoniaco se considera un parámetro importante para la formación de las comunidades metanogénicas. Se requiere $N\text{-}NH_4^+$ como la principal fuente de N para la mayoría de los metanógenos (Garcia et al., 2000), pero al mismo tiempo, los iones de amoníaco y el amoníaco libre pueden inhibir la metanogénesis (Yenigün and Demirel, 2013). El efecto del amoníaco sobre la dinámica de la población y la actividad de los metanógenos se ha descrito más detalladamente en digestores anaeróbicos a concentraciones promedio mucho más altas (generalmente >1 gL^{-1}) que las medidas en el influente de la línea de aguas (<0.05 gL^{-1}); sin embargo, se observaron patrones muy similares de sucesión de comunidades en respuesta al amoníaco. Estudios previos concluyeron que los metanógenos hidrogenotróficos son más tolerantes a los niveles de amoníaco que los metanógenos acetotróficos, y que *Methanosaeta*, acetoclásticos obligados, son los más sensibles al amoníaco (Karakashev et al., 2005; Yenigün and Demirel, 2013).

Es importante destacar que la abundancia relativa de *Nitrososphaera* aumentó de 1.05% en la Fase I a 6.74% en la Fase II, ya que es la arquea oxidante de amoníaco (AOA) identificada con mayor frecuencia en las EDARs urbanas (Limpiyakorn et al., 2013). Además, se encontraron fuertes correlaciones positivas (r>0.65) entre la abundancia relativa de estas AOA y la concentración de $N\text{-}NH_4^+$, TN y la ratio de eliminación de TN (NRR) (Table S11B, Chapter 2). Las bacterias oxidantes del amoníaco (AOB) se han considerado clásicamente como los organismos principales de la nitrificación en las EDARs (Yin et al., 2018); sin embargo, en las últimas décadas se ha establecido que las

AOA coexisten a menudo con las AOB en estos tipos de biorreactores (Limpiyakorn et al., 2013), dentro de un amplio rango de concentraciones de amoníaco (Yin et al., 2018), donde se ha demostrado recientemente una importante contribución en el proceso de nitrificación (Roy et al., 2017; Srithep et al., 2018). Los resultados de esta Tesis Doctoral mostraron que la abundancia relativa de filotipos relacionados con *Nitrososphaera* se vio favorecida por concentraciones crecientes de amoníaco en el influente, y se correlacionó concomitantemente con una mejor eliminación de TN (NRR), lo que respalda la relevancia de su papel en la oxidación del amoníaco.

8. Relaciones entre la abundancia relativa de las bacterias y arqueas con los REs de los PhACs estudiados en el sistema A^2O de la línea de aguas

En las últimas décadas se han realizado una gran cantidad de estudios sobre la influencia de las condiciones operacionales/ambientales y las concentraciones de materia orgánica y nutrientes en la estructura y diversidad de las comunidades microbiana presentes en los biorreactores de las EDARs (Ferrera and Sánchez, 2016; Tian and Wang, 2020; Xia et al., 2018). Asimismo, también se han realizado numerosos estudios sobre la capacidad de los diferentes sistemas de tratamiento de aguas residuales urbanas para eliminar/remover los PhACs (Grandclément et al., 2017; Tran et al., 2018; Verlicchi et al., 2012). Sin embargo, existen escasos estudios sobre la influencia de los PhACs en la diversidad de la comunidad microbiana y sobre la relación de la abundancia o presencia de ciertos grupos microbianos con la capacidad de biodegradación/biotransformación de los PhACs en los procesos de tratamiento de aguas residuales urbanas (Chen et al., 2017). La mayoría de estos estudios están realizado en sistemas a escala laboratorio y usando agua sintética con PhACs añadidos normalmente a concentraciones más elevadas que las concentraciones reales presentes en las aguas residuales urbanas (Jiang et al., 2017; Li et al., 2016; Rodriguez-Sanchez et al., 2017; Tiwari et al., 2019).

En esta Tesis Doctoral se han investigado los vínculos entre la abundancia relativa de los distintos grupos de bacterias y arqueas en la comunidad microbiana del fango activo y las eficiencias de remoción/eliminación (REs) de los distintos PhACs en el sistema A^2O de las Fases I y II. Para ello se utilizó igualmente el análisis MDS y BIO-ENV (Figure 3 y Table S12, Chapter 2). El análisis BIO-ENV reveló fuertes correlaciones entre la eliminación de PhACs y las abundancias relativas de ciertos grupos de bacterias y arqueas. En general, las tendencias de los vectores en el gráfico reflejan que los RE de la mayoría

de los PhACs analizados fueron más altos en la Fase II, con la excepción de la hidroclorotiazida.

En cuanto a las bacterias, al nivel taxonómico de familia se encontró que abundancias relativas de *Rhodobacteraceae* y *Sphingomonadaceae* se correlacionaron positivamente (r de Pearson r>0.75) con los REs de claritromicina, furosemida, bezafibrato y gemfibrozil, mientras que la abundancia relativa de *Comamonadaceae*, *Rhodocyclaceae* y *Caldilineaceae* se correlacinó positivamente (r=0.75-1.0) con los REs de 7 PhACs de diferentes grupos terapéuticos (ibuprofeno, naproxeno, claritromicina, furosemida, atenolol, bezafibrato y gemfibrozil) (Figure 3 y Table S12, Chapter 2). Por otro lado, la abundancia relativa de *Xanthomonadaceae*, *Chromatiaceae* y *Cytophagaceae* se correlacionó positivamente (r>0.90) con los REs de la hidroclorotiazida, único compuesto junto al sotalol que obtuvo significativamente mayor RE medio en la Fase I.

Los resultados obtenidos en esta Tesis Doctoral son consistentes con los de trabajos anteriores, a pesar de las diferentes condiciones operacionales y de concentración en el influente de estos estudios. En este sentido, las *Alfaproteobacterias* y las *Betaproteobacterias* se han relacionado a menudo con la eliminación biológica de PhACs en las EDARs basadas en diferentes tecnologías. Las abundancias relativas de poblaciones clasificadas dentro de la familia *Sphingomonadaceae* se encontraron anteriormente correlacionadas positivamente con los RE del antibiótico trimetoprima en un biorreactor CANON de eliminación de nitrógeno autótrofo (Rodriguez-Sanchez et al., 2017), dicha familia y dos géneros (*Sphingorhabdus* y *Novosphingobium*) fueron señalados como indicadores de condiciones favorables para la remoción de bezafibrato tanto en una EDAR a gran escala como a escala piloto (Wolff et al., 2018). Anteriormente se observó que el enriquecimiento en las comunidades de fangos activos de filotipos relacionados con varios géneros de *Comamonadaceae* y *Rhodocyclaceae* ocurría de manera concomitante con la eliminación de bezafibrato y claritromicina (Wolff et al., 2018), ibuprofeno (Li et al., 2016), y trimetoprima (Rodríguez-Sánchez et al., 2017). Diferentes taxones aislados pertenecientes a las familias *Comamonadaceae* y *Sphingomonadaceae* también suelen ser capaces de metabolizar/cometabolizar diferentes tipos de PhACs en condiciones de laboratorio, ya sea en cultivos puros o como parte de consorcios (Fortunato et al., 2016; Wang and Wang, 2016).

En cuanto a las arqueas, al nivel taxonómico de género se encontró que las abundancias relativas de *Methanoregula*, *Methanosphaera*, *Methanobrevibacter* y

Methanosaeta se correlacionaron positivamente (r>0.60) con los REs de acetaminofeno, ibuprofeno, naproxeno, claritromicina, atenolol, bezafibrato y gemfibrozil. Igualmente, la abundancia relativa de *Methanomethylovorans* se correlacionó positivamente (r>0.60) con los REs de ibuprofeno, naproxeno, claritromicina, atenolol, furosemida, bezafibrato y gemfibrozil. Asimismo, las abundancias relativas de *Nitrososphaera* y *Methanobacterium* se correlacionó (r=0.70-1.00) con los REs de furosemida y bezafibrato.

Debido a los escasos trabajos presente en la literatura que estudien la relación de eliminación/remoción de los PhACs junto con su abundancia relativa de arqueas no se han podido realizar suficientes comparaciones con bibliografía. No obstante, se ha observado la implicación de las arqueas en la biodegradación de contaminantes orgánicos como los hidrocarburos en el suelo y el agua (Krzmarzick et al., 2018). Asimismo, el género acetoclástico *Methanosaeta* aumentó significativamente su abundancia en suelos contaminados con diésel frente a suelos no contaminados (Krzmarzick et al., 2018) y también dominó la comunidad en una EDAR que trata aguas residuales industriales que contienen colorantes azoicos (Plumb et al., 2001). *Methanomethylovorans*, un metanógeno metilotrófico obligado capaz de utilizar metanol, metanotiol, metilaminas y sulfuro de dimetilo como sustratos (Lomans et al., 1999), se ha descrito como la arquea metanogénicas dominante en las EDARs que tratan altas concentraciones de antibióticos, señalando su implicación junto con bacterias sulfatorreductoras en las últimas etapas de biodegradación de estos compuestos en condiciones anaeróbicas (Deng et al., 2012). Anteriormente se propuso un papel similar de *Methanomethylovorans* en la degradación anaeróbica de tintes con grupos sulfonato en aguas residuales industriales (Plumb et al., 2001).

Mención aparte merecen los filotipos relacionados con las AOA *Nitrososphaera*, en el cual su abundancia relativa dentro de la comunidad de arqueas del sistema A^2O mostró correlaciones positivas con los RE de furosemida, bezafibrato y gemfibrozil (Figure 3 y Table S12, Chapter 2). Anteriormente se demostró que la eliminación de estos tres PhAC, entre otros, se vio obstaculizada significativamente después de la inhibición química de la actividad de amonio monooxigenasa en MBR a escala de laboratorio y biofiltros empaquetados con antracita (Maeng et al., 2013; Rattier et al., 2014). En la literatura, la participación de los nitrificantes en la eliminación de trazas de contaminantes orgánicos de las aguas residuales se ha atribuido principalmente a las AOBs (Kumwimba et al., 2019); sin embargo, en diferentes muestras de fangos activados de 10 plantas EDARs urbanas a

gran escala, Helbling et al. (2012) concluyeron que tanto la oxidación del amoníaco como la eliminación de PhACs se correlacionaron con la abundancia de transcripciones de *amoA* de arqueas, en lugar de bacterias.

9. Estructura y composición de Bacterias, Arqueas y Hongos en la digestión bifásica de la línea de fangos

Las comunidades microbianas desarrolladas en los digestores anaeróbicos se caracterizan por la presencia de una comunidad metabólica amplia y compleja de miembros anaeróbicos estrictos y facultativos de los dominios Bacterias y Arquea, lo que permite la biodegradación anaeróbica de diferentes compuestos orgánicos en un valioso compuesto energético como es el metano. En los sistemas de digestión anaeróbica, las bacterias son mayores en términos de cantidad y biodiversidad en comparación con las arqueas (Collins et al., 2006) y son las responsables de los tres primeros procesos metabólicos (hidrólisis, acidogénesis y acetogénesis) descritos anteriormente en la Figura I-10. Sin embargo, el paso final del proceso de metanogénesis se completa con la participación del dominio Arquea. Aunque los miembros del dominio Arquea tienen una abundancia y diversidad relativamente bajas en comparación con los miembros del dominio Bacteria, juegan un papel importante porque son los únicos responsables de la producción de metano.

En este sentido, mediante secuenciación masiva en paralelo (Illumina-sequencing) se obtuvo (97% de cut-off) un mayor número total de filotipos para las bacterias que para las arqueas (1341 PHYs de bacterias, 108 PHYs de arqueas y 246 PHYs de hongos). Asimismo, las bacterias obtuvieron unos valores de abundancia absoluta mayores que las arqueas en todos los digestores y fases (Table S5, Chapter 3). Solo se encontró únicamente diferencias significativas para la abundancia absoluta de arqueas entre fases del MD (Table S5, Chapter 3). La mayor abundancia absoluta de arqueas encontradas en el MD de la Fase I concuerda con la mayor tasa de recuperación de CH_4 (Table 1, Chapter 3), debido a una mejor eficiencia en la separación de los procesos hidrolisis/acidogénesis y acetogénesis/metanogénesis en la digestión bifásica de la Fase I. En general, las valores medios de abundancias absolutas por L de fango obtenidas mediante qPCR en los diferentes digestores y fases fueron inferiores a los reportados para digestores de fangos de EDARs medidos mediante qPCR y PCR digital por gotas (Jang et al., 2014; Kim et al., 2015 y Nettmann et al., 2010).

Destacar que los hongos solo fueron identificados en la Fase I en concordancia con los datos obtenidos mediante qPCR en la línea de aguas. Hasta la fecha, se dispone de

escasa información sobre los organismos eucariotas anaeróbicos (hongos). Se conoce que algunos hongos contribuyen a la degradación de la materia orgánica en condiciones anaeróbicas, por lo que podrían estar implicados en la hidrólisis de materia orgánica en la digestión anaeróbica de fangos de EDARs (Castellano-Hinojosa et al., 2018a).

Entre las fases y tipo de digestores se detectaron diferencias importantes en la estructura de la comunidad bacteriana a nivel de Filo, Clase, Orden y Familia (Figure 1, Figure S9 y Table S7, Chapter 3). Los filos dominantes en el AcD fueron *Proteobacteria* en Fase I y *Firmicutes* en Fase II, mientras que en el MD los filos *Bacteroidetes* y *Proteobacteria* dominaron en ambas fases, seguidos de *Bacteroidetes* y *Proteobacteria*. Estos cuatro filos bacterianos mencionados anteriormente (*Bacteroidetes, Chloroflexi, Firmicutes* y *Proteobacteria*) son los grupos principales detectados en la AD mesofílica de fangos de depuradora mediante enfoques metagenómicos basados en secuenciación de alto rendimiento (HTS) (McIlroy et al., 2017; Nelson et al., 2011; Wu et al., 2017; Yang et al., 2014), y comprenden microorganismos que se sabe que participan en una o más de las etapas del proceso de la AD (hidrólisis, acidogénesis y acetogénesis) (Westerholm and Schnürer, 2019; Xia et al., 2016). Finalmente, a nivel de familia los grupos dominantes en el AcD fueron *Rikenellaceae* en fase I (11.13%) y *Turicibacteraceae* en fase II (25.00%), mientras que *Rikenellaceae* prevaleció en el MD en ambas fases experimentales (33.8% en fase I y 18.33% en Fase II; Figure S9, Chapter 3).

En relación a las arqueas, el 99.9% de las secuencias se clasificaron como *Euryarchaeota* (Figure 3, Chapter 3). A nivel de género, también se encontraron grandes diferencias en la estructura de la comunidad de arqueas metanógenas entre los digestores y las fases. En este sentido, cada fase presentó un género de metanógenas dominante; en el AcD el género fue *Methanolinea* (hidrogenotroficas) en fase I y la *Methanosarcina* (versátil) en fase II, mientras que el MD tubo codominancia de dos géneros en cada fase; *Methanospirillum* y *Methanolinea* (hidrogenotroficas) en Fase I y *Methanospirillum* y *Methanosarcina* en Fase II. Se conoce que *Euryarchaeota* abarca la mayoría de los metanógenos, junto con arqueas reductoras de sulfato, termófilas extremas y halófilas (Oren, 2019).

10. Relaciones entre las variables operacionales y de rendimiento con la abundancia relativa de las bacterias y arqueas de la digestión bifásica

Para ilustrar mejor las diferencias observadas en la comunidad de bacterias y arqueas de los digestores AcD y MD durante las dos fases experimentales, las muestras fueron

ordenadas mediante un análisis MDS (Figure 2, Chapter 3) de acuerdo con las abundancias relativas a nivel de filo y familia para las bacterias y a nivel de género para las arqueas, junto con los parámetros operativos y de rendimientos del proceso de digestión.

En relación a las bacterias, se pudieron identificar relaciones positivas interesantes entre los indicadores de la eficiencia de producción de metano (VSR, composición de CH_4 y la ratio de recuperación de CH_4) y diferentes grupos de familias de bacterias (*Dethiosulfovibrionaceae*, *Rikenellaceae*, *Syntrophaceae*, y *Turicibacteraceae*) así como relaciones negativas con otro grupo de familias (Figure 2B y C, Table S9, Chapter 3). Con los datos operacionales pudimos identificar que la separación entre fases de la digestión anaeróbica solo fue efectiva en la Fase I, siendo la ratio VFA/ALK un importante indicador de la producción de VFA y de la efectividad de la separación. En este sentido, los filotipos dominantes en el AcD cambiaron de *Proteobacteria* en Fase I a *Firmicutes* en Fase II; en el cual, se identificaron que las familias *Cytophagaceae*, *Lachnospiraceae* y *Xanthomonadaceae* se correlacionaron positivamente con la ratio VFA/ALK y fueron más abundantes en la Fase I (Figure S9 y Figure 2, Chapter 3). Los miembros de las familias *Cytophagaceae* y *Lachnospiraceae* son bien conocidos por su papel clave en la hidrólisis de varios tipos de macromoléculas, y pertenecientes a la familia *Lachnospiraceae* generan VFA como acetato, propionato y butirato a través de la fermentación (Biddle et al., 2013; McBride et al., 2014). Por otro lado, las *Xanthomonadaceae* son principalmente bacterias estrictamente aeróbicas; sin embargo, a menudo se han encontrado en abundancias relativas significativas en biorreactores de AD, y sus funciones en estos sistemas aún no están bien definidas (Hao et al., 2016).

Después de los procesos de hidrólisis y acidogénesis que ocurren principalmente en el AcD de una digestión bifásica, los compuestos orgánicos simples como el acetato, hidrógeno y CO_2 son producidos en el MD a partir de los VFA generados por el AcD. Estos sustratos de las arqueas metanogénicas son producidos a través de la actividad de las bacterias homoacetógenicas y acetógenas sintróficas (Figure I-10) que pertenecen en su mayoría a *Firmicutes* y *Proteobacteria*, aunque ciertos miembros de los filos *Actinobacterias*, *Bacteroidetes*, Ca. Cloacimonetes, *Chloroflexi* y *Synergistetes* también se han observado en reacciones metabólicas sintróficas con las arqueas metanogénicas (Narihiro et al., 2015; Westerholm and Schnürer, 2019). Sin embargo, no se encontraron correlaciones positivas entre la abundancia relativa de familias de bacterias homoacetógenicas y acetógenas sintróficas detectadas en las dos fases con las tasas de

recuperación de CH_4 (Tabla S9, Chapter 3), lo que sugiere que este paso fue realizado por una comunidad funcionalmente redundante y no dependió de la abundancia relativa de poblaciones particulares. No obstante, la abundancia relativa de *Dethiosulfovibrionaceae* (S*ynergistetes*), *Syntrophaceae* (*Deltaproteobacteria*) y *Rikenellaceae* (*Bacteroidetes*) se correlacionaron positivamente con el VSR y las tasas de recuperación de CH_4, y al mismo tiempo se vieron favorecidas por SRT más largos (Figuras 2B y Tabla S9, Chapter 3). La familia *Syntrophaceae* fue el único grupo de acetógenos sintróficos conocidos que alcanzó una abundancia relativa promedio >1% en los digestores, y más específicamente en muestras del MD durante la Fase II. Finalmente, destacar que los miembros de la familia *Rikenellaceae* son consideradas como bacterias anaeróbicas mesófilas capaces de fermentar carbohidratos o proteínas, generando propionato, succinato, acetato, alcoholes, H_2 o CO_2 como productos finales (Graf, 2014; Su et al., 2014). *Rikenellaceae* fue compartida por todas las muestras (Figura S7) y fue el taxón más abundante a nivel familiar en el MD de las dos fases, lo que apunta a tener un rol importante en el rendimiento de la digestión bifásica. Los miembros de este grupo se han encontrado previamente con una abundancia relativa alta en EDARs con procesos anaeróbicos y digestores anaeróbicos (Hao et al., 2016; Yi et al., 2014). La abundancia relativa de *Rikenellaceae* se correlacionó negativamente con OLR, de acuerdo con estudios anteriores (Hao et al., 2016).

En relación a las arqueas, los géneros *Methanospirillum* and *Methanoculleus* se identificaron con mejores eficiencias de producción de metano, mientras que el dominio de *Methanolinea* y *Methanobrevibacter* se identificaron con una menor eficiencia de producción de metano (Table S11, Chapter 3). Se detectaron competencias entre los géneros *Methanosaeta* y *Methanosarcina* a lo largo del período experimental (Figura 2D, Chapter 3). La competencia entre estos dos géneros ha sido ampliamente reportada en estudios previos (Castellano-Hinojosa et al., 2018a) y se explica por diferencias en sus respectivas afinidades por acetato y tasas de utilización, que favorecen el crecimiento de *Methanosaeta* en ambientes con bajas concentraciones de acetato, bajos VFA o bajos OLR (Xu et al., 2018; Yang et al., 2014). Es importante destacar que, en el AcD, el género *Methanolinea* prevaleció en la Fase I, pero fue desplazada por *Methanosarcina* en la Fase II (Figura 3, Chapter 3). Este cambio se relacionó con una mayor producción de biogás y metano en Fase II (Table 1A, Chapter 3), haciendo que la separación entre las etapas hidrólisis/acidogénesis y acetogénesis/metanogénesis de la digestión bifásica solo fuera efectiva en Fase I y parcial en Fase II, tal y como se ha discutido anteriormente. En

resumen, los resultados de la secuenciación por Illumina mostraron que la generación de metano en la digestión bifásica fue impulsada principalmente por la actividad biológica de los metanógenos reductores de dióxido de carbono que utilizan hidrógeno o formiato como donantes primarios de electrones (*Methanolinea* y *Methanospirillum*), o metanógenas versátiles como *Methanosarcina*, capaces de generar CH_4 por vías acetoclásticas, hidrogenoclásticas y/o metilotróficas (Oren, 2019). El estado estacionario en los procesos AD mesófila se ha relacionado clásicamente con la prevalencia de metanógenos acetoclásticos; sin embargo, también se ha reportado un funcionamiento estable en sistemas dominados por *Methanospirillum* (revisado por Saha et al. 2020).

11. Relaciones entre la abundancia relativa de las bacterias y arqueas con las REs de los PhACs estudiados en la digestión bifásica

Hoy en día, la relación entre la biotransformación/biodegradación de los PhACs y la actividad microbiana en el proceso de AD todavía está poco investigada. En este sentido Stasinakis, (2012) indicó que la abundancia de microorganismos capaces de biodegradar PhACs es un factor crucial para su eliminación en el proceso de AD basado en la mayor biodegradación de diclofenaco, diazepam y estrógenos durante la aclimatación de la biomasa (Carballa et al., 2007, 2006). Asimismo, Gonzalez-Gil et al. (2018) teorizó a partir de sus resultados que el proceso de metanogénesis juega un papel muy importante en la eliminación de los PhACs durante el proceso de AD. Esta teoría concuerda con lo expuesto por Ghattas et al. (2017), que propusieron una gran variedad de tipos de biorreacciones de microcontaminantes orgánicos por microorganismos metanógenos. En esta Tesis Doctoral se ha realizado un análisis MDS para encontrar relaciones significativas entre los REs de 6 PhACs que se eliminaron en todas las muestras de los dos digestores y en ambas fases y la abundancia relativa de bacterias y arqueas en los AcD y MD (Figure 4, Chapter 3).

Los resultados mostraron que las REs de naproxeno y trimetoprima se correlacionan positivamente con la abundancia relativa de *Cythophagaceae*, con varias familias de bacterias acetógenas de la clase *Clostridia* y el género de arquea metanógena versátil *Methanosarcina.* Asimismo, la RE de fenofibrato se correlaciona positivamente con la abundancia relativa de la familia *Rhodobacteraceae*, de forma análoga a lo observado en la línea de aguas con los compuestos de la misma familia terapéutica (bezafibrato y gemfibrozil). Las especies de esta familia a menudo tienen capacidades para la degradación aerobia y anaerobia de compuesto orgánicos; no obstante, su influencia en la degradación

de compuestos orgánicos traza en los sistemas de digestión anaerobia está muy poco explorada.

Las vías de biotransformación o biodegradación seguidas por estos y otros PhAC en la AD de fangos de EDARs urbanas están muy poco exploradas; sin embargo, existen algunos hallazgos reportados en estudios anteriores que están de acuerdo con las observaciones presentadas en esta Tesis Doctoral. La biotransformación de naproxeno y trimetoprima en condiciones anaeróbicas está bien caracterizada y tiene lugar mediante O-desmetilación de grupos fenil-metil-éter (Ghattas et al., 2017). Wolfson y col. (2019, 2018) caracterizaron la diversidad de dos consorcios capaces de O-desmetilar naproxeno en condiciones metanogénicas y reductoras de sulfato, observándose un enriquecimiento de bacterias fermentativas y acetogénicas, en su mayoría pertenecientes al filo *Bacteroidetes* y/o la clase *Clostridia*; además, estos autores postularon que tanto los metanógenos acetoclásticos como los hidrogenotróficos contribuyen a convertir los grupos metilo del naproxeno en metano, ya sea utilizando acetato fácilmente o mediante una asociación con oxidantes de acetato sintróficas, respectivamente. Curiosamente, no se encontró superposición de la estructura de la comunidad bacteriana entre los dos consorcios, ya que las familias dominantes diferían dependiendo de la fuente del inóculo (fangos de AD de EDARs o sedimentos marinos anóxicos), lo que respalda que la biotransformación del naproxeno debería depender de taxones funcionalmente redundantes. Del mismo modo, Liang et al. (2019) obtuvieron que la biotransformación de trimetoprima en condiciones reductoras de sulfato a través de O-desmetilación y una mayor degradación del derivado hidroxilado, realizado a nivel de clase por consorcio de *Bacteroidia, Sphingobacteriia*, (*Bacteroidetes*), *Clostridia* (*Firmicutes*), y *Deltaproteobacteria* (*Proteobacteria*). Las correlaciones positivas encontradas en esta Tesis Doctoral entre los RE de naproxeno y trimetoprima y las abundancias relativas de *Cytophagaceae* (*Bacteroidetes*), los acetógenos de la clase *Clostridia* (es decir, *Christensenellaceae, Halanaerobiaceae, Peptostreptococcaceae* y *Ruminococcacee*) y el género *Methanosarcina* (Tabla S13, Chapter 3) están de acuerdo con los estudios antes mencionados y sugieren que patrones análogos de biotransformación podrían tener lugar en la digestión bifásica mesófila. En este sentido, resaltar que el naproxeno y la trimetoprima incrementó su RE medio en el sistema A^2O-IFAS de la línea de aguas; en el cual, el incremento de mayores condiciones anaeróbicas proporcionado por la biomasa adherida a los soportes podría ser un factor

determinante en la mejora de las REs (Table S5, Chapter 4). Sin embargo, confirmar estas hipótesis requerirá esfuerzos de investigación adicionales.

Las fluoroquinolonas (antibióticos) tienden a adsorberse en los fangos de las EDARs, además estos están considerados compuestos de difícil biodegradabilidad (Rusch et al., 2019). No obstante, estos PhACs experimentan una variedad de transformaciones biológicas en el medio ambiente, mediadas tanto por hongos como por bacterias. Hasta la fecha se han identificado más de 20 productos de transformación para el ciprofloxacino (Rusch et al., 2019). Se supone que, debido a la gran cantidad de reacciones posibles, es más probable que los consorcios de especies mixtas estén involucrados en la biodegradación de fluoroquinolonas en el medio ambiente (Rusch et al., 2019). En consecuencia, en el presente estudio, el ofloxacino mostró fuertes correlaciones positivas con un gran número de familias bacterianas evolutivamente dispersas (Figura 4 y Table S13, Chapter 3).

VI. CONCLUSIONES/CONCLUSIONS

CONCLUSIONES

De acuerdo con los resultados obtenidos en la presente Tesis Doctoral, así como la revisión bibliográfica llevada a cabo, se presentan las siguientes conclusiones finales que dan respuesta a los objetivos principales propuestos. Para ello, se han estudiado dos tecnologías diferentes en línea de aguas durante tres fases experimentales (sistema A^2O en Fase I y II, y sistema A^2O-IFAS en Fase III), y un sistema avanzado en línea de fangos (digestión bifásica) durante dos fases experimentales (Fase I y II).

- **Conclusiones relativas a la línea de aguas, sistema A^2O y sistema A^2O-IFAS:**

1. El sistema convencional A^2O obtuvo mejores rendimientos de eliminación de materia orgánica y nutrientes (nitrógeno total y fósforo) cuando operó con el doble de concentración de biomasa suspendida (MLSS), con menor F/M ratio y con una mayor COD/TN ratio. Estas condiciones se alcanzaron en la Fase II. La transformación del sistema convencional A^2O en el sistema avanzado A^2O-IFAS al introducir el relleno plástico AnoxKaldnes K5 con un factor de relleno del 50% en las cámaras aeróbicas mejoró significativamente las ratios de eliminación de materia orgánica y nutrientes con respecto al sistema A^2O homólogo.

2. La mejora de los rendimientos del sistema A^2O-IFAS se produce con un menor coste operacional, debido a que el sistema operó con menor recirculación interna y externa (MLR y RAS), con menor concentración de biomasa suspendida (MLSS), con menor tiempo de retención hidráulico (TRH), así como con una mayor carga aplicada de nitrógeno (NLR) y de fósforo (PLR).

3. Según el balance másico global realizado en la planta piloto para los 27 PhACs seleccionados, concluimos que la eliminación se debe principalmente a procesos de biodegradación/biotransformación, ya que el porcentaje de remoción global por sorción en línea de aguas es inferior a 1.2% en las dos Fases de estudio. Únicamente la paroxetina y el fenofibrato en las Fases I y II, así como los fármacos ofloxacino y sotalol en la Fase I y la codeína en Fase II fueron removidos mediante sorción en más de un 5%.

4. La eficiencia de eliminación (RE) de los PhACs ibuprofeno, naproxeno, claritromicina, bezafibrato y gemfibrozil, fue mayor en el sistema A^2O cuando éste operó con el doble de concentración de MLSS y menor F/M ratio (Fase II frente a

Fase I). De los 19 PhACs que pudieron ser evaluados en función de su REs, el sistema A^2O-IFAS obtuvo REs más elevadas para 8 PhACs (ibuprofeno, ketoprofeno, naproxeno, claritromicina, trimetoprima, atenolol, bezafibrato y gemfibrozil) con respecto al sistema A^2O de la Fase I, y 5 PhACs (diclofenaco, ibuprofeno, naproxeno, trimetoprima y sotalol) con respecto al sistema A^2O de la Fase II.

5. Los parámetros operacionales que más influyeron en la mejora significativa de las REs entre las tres Fases (Fases I, II y III) fueron principalmente la mayor concentración de biomasa (MLSS) y el menor valor del F/M ratio para los PhACs ibuprofeno, naproxeno, ketoprofeno, gemfibrozil, atenolol, claritromicina y bezafibrato. Asimismo, altas cargas de $N\text{-}NH_4^+$, junto con altos rendimientos de eliminación de nitrógeno, materia orgánica y fósforo (NRR, ORR and PRR) se asociaron a incrementos de las REs del naproxeno, claritromicina y bezafibrato.

6. El número de PhACs vertidos mediante el efluente secundario que son susceptibles de causar un efecto tóxico al medio acuático se redujo de 12 (acetaminofén, diclofenaco, ibuprofeno, naproxeno, claritromicina, ofloxacino, sulfametoxazol, trimetoprima, propanolol, furosemida, gemfibrozil y lorazepam) en el sistema A^2O a 7 cuando se operó con el sistema A^2O-IFAS (diclofenaco, ibuprofeno, ofloxacino, sulfametoxazol, trimetoprima, furosemida, gemfibrozil).

7. En el sistema A^2O se encontraron correlaciones negativas entre la abundancia absoluta de arqueas totales y hongos con la abundancia absoluta de *Mycolata*.

8. El análisis MDS indicó importantes correlaciones positivas entre la abundancia relativa de varias familias de bacterias con las concentraciones de MLSS en el biorreactor y las concentraciones de COD, BOD_5 y $N\text{-}NH_4^+$ en el influente.

9. Los resultados de esta Tesis Doctoral mostraron que la abundancia relativa de filotipos relacionados con *Nitrososphaera* se vio favorecida por concentraciones crecientes de amoníaco en el influente, y se correlacionó concomitantemente con una mejor eliminación de TN (NRR), lo que respalda la relevancia de su papel en la oxidación del amoníaco.

10. El análisis BIO-ENV y MDS estableció vínculos robustos entre los REs de varios PhACs (acetaminofeno, ibuprofen, ketoprofeno, naproxeno, claritromicina, trimetoprima, atenolol, furosemida, hidroclorotiazida, bezafibrato, and gemfibrozil)

y la abundancia relativa de familias de *Bacteria* (*Rhodobacteraceae*, *Sphingomonadaceae*, *Comamonadaceae*, *Rhodocyclaceae*, *Caldilineaceae*, *Chitinophagaceae* y *Flavobacteriaceae*) y géneros de *Arquea* (*Methanobrevibacter*, *Methanoregula*, *Methanosaeta*, *Methanosphaera*, *Methanomethylovorans* y *Nitrososphaera*).

- **Conclusiones relativas a la línea de fangos, digestión bifásica:**

1. La mejor eficiencia de los procesos de hidrólisis/acidogénesis en el digestor acidogénico (AcD) relacionada con una mayor producción de ácidos grasos volátiles (VFA) y con una mayor abundancia absoluta de arqueas totales en el digestor metanogénico (MD), determinaron una mayor tasa de recuperación de CH_4 en la Fase I con respecto a la Fase II, esto es, en la fase en la que se operó con menor tiempo de retención de sólidos, SRT, en ambos digestores. En función de la producción de VFA y metano en los digestores AcD y MD, así como los datos de la abundancia relativa y absoluta de la comunidad microbiana, la digestión bifásica solo fue efectiva en la Fase I.

2. El tiempo de retención de sólidos (SRT) empleado en el MD de la Fase II incrementó significativamente las REs de 7 PhACs (carbamazepina, claritromicina, codeína, gemfibrozil, ibuprofen, lorazepam y propanolol) en comparación con la Fase I.

3. Se identificaron correlaciones positivas entre los indicadores de la eficiencia de producción de metano (VSR, composición de CH_4 y la ratio de recuperación de CH_4) y diferentes familias bacterianas (*Dethiosulfovibrionaceae*, *Rikenellaceae*, *Syntrophaceae*, y *Turicibacteraceae*). La familia *Rikenellaceae* fue compartida por todas las muestras y fue el taxón más abundante en el MD de las dos fases, indicando su rol en la interacción con las arqueas metanogénicas, influyendo así en el rendimiento de la digestión bifásica.

4. El análisis MDS permitió establecer vínculos robustos entre las REs de varios PhACs (atenolol, fenofibrato, ketoprofeno, naproxeno, ofloxacino y trimetoprima) y la abundancia relativa de grupos de bacterias y arqueas, poniendo de manifiesto la relevancia de los procesos de biodegradación/biotransformación de estos compuestos xenobióticos en digestión bifásica.

CONCLUSIONS

Based on the results obtained in this PhD Thesis, as well as the bibliographic review carried out, the following final conclusions that respond to the main proposed objectives are presented. By this means, two different technologies have been studied in the water line during three experimental phases (A^2O system in Phase I and II, and A^2O-IFAS system in Phase III), and one advanced system in sludge line (two-stated MAD) during two experimental phases (Phase I and II).

- **Conclusion related to the water line, A^2O systems and A^2O-IFAS system:**

1. The conventional A^2O system obtained better removal efficiencies of organic matter and nutrients (total nitrogen and phosphorus) when it operated with twofold the concentration of suspended biomass (MLSS), with a lower F/M ratio and a higher COD/TN ratio in Phase II. The transformation of the conventional A^2O system into the advanced A^2O-IFAS system by filling the aerobic basins (50% filling ratio) with the carrier AnoxKaldnes K5, significantly improved the elimination rates of organic matter and nutrients with respect to the homologous A^2O system.

2. The performance improvement of the A^2O-IFAS system occurs with a lower operational cost, because the system operated with less internal and external recirculation (MLR and RAS), with lower concentration of suspended biomass (MLSS), with less HRT, and with a higher nitrogen loading rate (NLR) and phosphorus loading rate (PLR).

3. According to the global mass balance carried out in the pilot-scale plant for the 27 selected PhACs, we conclude that the removal is mainly done by the biodegradation/biotransformation processes, since the percentage of global removal by sorption in the water line is less than 1.2% in the two phases. Only paroxetine and fenofibrate in Phases I and II, as well as the PhACs ofloxacin and sotalol in Phase I and codeine in Phase II were removed by sorption in more than 5%.

4. The removal efficiency (RE) of the PhACs ibuprofen, naproxen, clarithromycin, bezafibrate and gemfibrozil, were higher in the A^2O system when it operated with twofold the concentration of MLSS and a lower F/M ratio (Phase II versus Phase I). From the 19 PhACs that was evaluated based on their REs, the A^2O-IFAS system obtained higher REs for 8 PhACs (ibuprofen, ketoprofen, naproxen, clarithromycin,

trimethoprim, atenolol, bezafibrate and gemfibrozil) compared to the A^2O system operated in Phase I, and 5 PhACs (diclofenac, ibuprofen, naproxen, trimethoprim, and sotalol) compared to the A^2O system operated in Phase II.

5. The operational parameters that most influenced the significant improvement of the REs among the three Phases (Phases I, II and III) were mainly the higher biomass concentration (MLSS) and the lower value of the F/M ratio for the PhACs ibuprofen naproxen, ketoprofen, gemfibrozil, atenolol, clarithromycin, and bezafibrate. Likewise, high influent concentration of $N\text{-}NH_4^+$, with high removal efficiencies of total nitrogen, organic matter and phosphorus (NRR, ORR and PRR) increased the REs of naproxen, clarithromycin and bezafibrate.

6. The number of PhACs discharged through secondary effluent that can produce an aquatic environmental ecotoxic effect was reduced from 12 (acetaminophen, diclofenac, ibuprofen, naproxen, clarithromycin, ofloxacin, sulfamethoxazole, trimethoprim, propranolol, furosemide, gemfibrozil and lorazepam) in the A^2O system to 7 when the pilot-scale plant operated with the A^2O-IFAS system (diclofenac, ibuprofen, ofloxacin, sulfamethoxazole, trimethoprim, furosemide, gemfibrozil).

7. In the A^2O system, negative correlations were found between the absolute abundance of archaea and fungi with the absolute abundance of Mycolata.

8. The MDS analysis indicated important positive correlations between the relative abundance of various families of *Bacteria* with the concentrations of MLSS in the bioreactor and the concentrations of COD, BOD_5 and $N\text{-}NH_4^+$ in the influent.

9. The results of this Doctoral Thesis showed that the relative abundance of *Nitrosophaera*-related phylotypes' was favored by increasing concentrations of ammonia in the influent, and was concomitantly correlated with better TN removal (NRR), supporting the relevance of its role in the nitrification process.

10. BIO-ENV and MDS analysis established robust interlinkages between the REs of several PhACs (acetaminophen, ibuprofen, ketoprofen, naproxen, clarithromycin, trimethoprim, atenolol, furosemide, hydrochlorothiazide, bezafibrate, and gemfibrozil) with shifts of the relative abundances of different families of *Bacteria* (*Rhodobacteraceae, Sphingomonadaceae, Comamonadaceae, Rhodocyclaceae,*

Caldilineaceae, Chitinophagaceae and *Flavobacteriaceae*) and the genera of *Archaea* (*Methanobrevibacter, Methanoregula, Methanosaeta, Methanosphaera, Methanomethylovorans* and *Nitrososphaera*).

- **Conclusion related to the sludge line, two-stage MAD**

1. The better efficiency of the hydrolysis/acidogenesis processes in the AcD related to a higher production of VFA and higher absolute abundance of total Archaea in the MD, determined a higher CH_4 recovery rate in Phase I compared to Phase II. Based on the production of VFA and methane in the AcD and MD digesters, and the relative and absolute abundance of the microbial community, biphasic digestion was only effective in Phase I.

2. The SRT used in the DM of Phase II increased the REs of 7 PhACs (carbamazepine, clarithromycin, codeine, gemfibrozil, ibuprofen, lorazepam and propranolol) compared to Phase I.

3. Positive correlations was identified between the methane production efficiency variables (VSR, CH_4 composition and the CH_4 recovery rate), and different groups of bacteria families (*Dethiosulfovibrionaceae, Rikenellaceae, Syntrophaceae,* and *Turicibacteraceae*). Family *Rikenellaceae* was shared by all samples and was the most abundant taxon at the family level in the MD of the two phases, which pointing to a key role of their interactions with methanogenic archaea for the performance of the two-stage MAD plant.

4. The MDS analysis allowed to establish robust interlinkages among the REs of several PhACs (atenolol, fenofibrate, ketoprofen, naproxen, ofloxacin and trimethoprim) with the relative abundance of groups of bacteria and archaea, showing their potential relationship with the biodegradation/biotransformation processes of these xenobiotic compounds in the two-stage MAD.

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IV. RESULTS-CHAPTER 1

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