Role of methanogenesis on the biotransformation of organic micropollutants during anaerobic digestion

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ABSTRACT

Several studies showed that some organic micropollutants (OMPs) are biotransformed during anaerobic digestion (AD). Yet, most of them aim at reporting removal efficiencies instead of understanding the biotransformation process. Indeed, how each of the main AD stages (i.e., hydrolysis, acidogenesis, and methanogenesis) contribute to OMP biotransformation remains unknown. This study focuses on investigating the role of methanogenesis, the most characteristic step of AD, to OMP removal. More specifically, the sorption and the biotransformation of 20 OMPs by methanogenic biomass were analyzed determining their concentrations in both liquid and solid phases. Sorption onto methanogenic biomass displayed a similar behavior as reported for digested sludge. Most of the OMPs were biotransformed to a medium extent (35-70%) and only sulfamethoxazole was completely removed. Comparing these results with those reported for the complete AD process, methanogenesis was proven to play a key role, accounting for more than 50% of the OMP biotransformation (except for roxithromycin) during AD. An increase in the organic loading rate from 1 to 2 g COD/L d, typical loads employed in sewage sludge anaerobic digesters, did not exert a clear cometabolic effect on the OMPs biotransformation. It is hypothesized that biotransformation occurs in both liquid and solid phases because no link between the partition coefficient (*Kd*) and the overall biotransformation efficiency was found. These findings allow a better understanding of the OMPs fate under anaerobic conditions, which is necessary to design efficient biological mitigation strategies.

Keywords: biotransformation, cometabolism, partition coefficient, pharmaceuticals, sewage sludge.

1. INTRODUCTION

The increasing presence of organic micropollutants (OMPs), such as pharmaceuticals, hormones, pesticides, household, and industrial chemicals in the environment is an issue of emerging concern due to their potential harm to ecosystems and public health (Luo et al., 2014). Indeed, research in this field and particularly dealing with the removal of pharmaceuticals in sewage treatment plants (STPs) (Qian et al., 2015) has experienced an exponential growth since the late 1990s (Daughton, 2016). Yet, studies dealing with OMPs fate in the sludge line of STPs are still very limited and no clear mitigation strategies are proposed.

Anaerobic digestion (AD), the main stabilization process of sewage sludge, has a limited biological capacity to remove OMPs (Stasinakis, 2012). Therefore, residual OMP concentrations can routinely be detected in digested sludge at levels above 1 mg/kg (Golet et al., 2003; Gonzalez-Gil et al., 2016; Langdon et al., 2011). A wide variety of OMPs was also found at lower concentrations (Petrie et al., 2015; Stasinakis, 2012), favoring the potential appearance of negative synergistic effects on the environment (Petrie et al., 2015). The capacity of OMP biotransformation of the anaerobic process should be maximized to fulfill the upcoming legislation limits of OMPs in biosolids (Inglezakis et al., 2014) pursuing a safe application of sludge in agricultural fields (Chen et al., 2014). To achieve this, it is essential to move from merely monitoring the removal efficiencies to understanding the factors influencing the fate of OMPs during AD.

Some authors suggest that the two main mechanisms affecting the fate of OMPs, sorption, and biotransformation, could be interrelated because sorption could modify the

biotransformation rates and the bioavailability of OMPs during AD (Barret et al., 2010c). The partition coefficient (*Kd*) is suitable to predict the distribution between phases and quantify the sorption of OMPs in solid matrices (Carballa et al., 2008). However, the concentrations of OMPs in the solid phase of digested sludge have been rarely measured (Petrie et al., 2015), leading to little information about their *Kd* values and the sorption role during AD.

Biotransformation is likely to occur through cometabolism (Delgadillo-Mirquez et al., 2011; Fernandez-Fontaina et al., 2016; Fischer and Majewsky, 2014; Plósz et al., 2010), due to the low concentrations of OMPs in comparison with the main growth substrate. This biotransformation process is influenced by the physicochemical properties of the OMPs, the microbial diversity, the enzymatic activities, and the environmental and operational parameters. Most studies about AD have focused on evaluating the effect of temperature, sludge retention time (SRT) and organic loading rate (OLR) on the removal of OMPs (Barret et al., 2010a; Carballa et al., 2007; Gonzalez-Gil et al., 2016; Malmborg and Magnér, 2015; Paterakis et al., 2012; Samaras et al., 2014; Zhou et al., 2017), concluding that these parameters are only relevant for few compounds. Nonetheless, little is known about other factors, such as the microbial population composition and the cometabolism linked to specific enzymes, and their relative significance on the biotransformation of OMPs during AD.

How microbial diversity affects the transformation of OMP is difficult to determine as there is a huge variety of microorganisms involved in the multistep AD process. Based on their physiology, nutritional needs, growth kinetics and sensitivity to environmental conditions, they can be classified into two main groups: acid-forming and methane-forming

microorganisms. The influence of each group on the fate of OMPs has not been addressed in depth, although there are a few evidences about the capacity of methanogens to biotransform some OMPs (Cetecioglu et al., 2016; Chang et al., 2005; Lahti and Oikari, 2011; Veetil et al., 2012).

The objective of this study is to ascertain the contribution of the methanogenic step to the removal of OMPs during the overall AD process, which is still considered a black-box in terms of OMPs fate. To this aim, the biotransformation capacity of methane-forming microorganisms was evaluated for a set of 20 OMPs with different physicochemical properties, paying special attention to the influence of the OLR and the phase partitioning.

2. MATERIALS AND METHODS

2.1 Selected organic micropollutants

The 20 selected OMPs present a wide variety of physicochemical characteristics and applications (Table S1) and an environmentally relevant occurrence in sewage sludge (Gonzalez-Gil et al., 2016; Paterakis et al., 2012; Stasinakis, 2012). They comprise three musk fragrances, galaxolide (HHCB), tonalide (AHTN) and celestolide (ADBI); three anti-inflammatories, ibuprofen (IBP), naproxen (NPX) and diclofenac (DCF); four antibiotics, sulfamethoxazole (SMX), trimethoprim (TMP), erythromycin (ERY) and roxithromycin (ROX); three neurodrugs, fluoxetine (FLX), carbamazepine (CBZ) and diazepam (DZP); four endocrine disrupting compounds from daily life products, bisphenol A (BPA), triclosan (TCS), 4-octylphenol (OP) and 4-nonylphenol (NP); and three hormones, estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2).

2.2 Methanogenic reactors

Two continuously stirred (IKA RW20, 150 rpm) lab-scale methanogenic reactors with a total volume of 15 L (liquid volume of 14 L) were operated under mesophilic conditions (37°C). Both reactors were inoculated with biomass (15-20 g VSS/L) from a nearby mesophilic sewage sludge digester and were operated semi-continuously by once-a-day manual feeding and withdrawal. The feeding consisted of a synthetic mixture of volatile fatty acids (VFA), acetic (HAc), propionic, and butyric acid in a ratio of 2:1:1 (COD basis) to limit the hydrolytic and acidogenic steps and to promote the symbiosis between acetogens and methanogens (Lin et al., 1986). Nitrogen (0.6 g NH_4Cl /L), phosphorous (0.4 g KH_2PO_4/L) , and trace concentrations of boron and metals (Fe, Ca, Mg, Cr, Co, Cu, Mn, Mo, Ni, Se, Zn) were added to the synthetic mixture since they are required for the microbial growth (Angelidaki and Sanders, 2004). The pH of the feeding was adjusted to 6-7 with NaOH. $NaHCO_3$ (5-10 g/L) was added to provide sufficient alkalinity. A more detailed description of the feeding characteristics is provided in Table S3.

The operation of the first methanogenic reactor (MR1) lasted 130 d (Fig. S1). During the start-up (25 d), a moderate OLR (0.5 g COD/L d) and high hydraulic retention time (HRT) (20 d) were applied to adapt the inoculum to the new conditions. Subsequently, the OLR was increased to 2 g COD/L d and the HRT was reduced to 10 d according to Rubio-Loza and Noyola (2010). These conditions were maintained for more than 1 month but the reactor operation being unsteady, the OLR was reduced to 1 g COD/L d (HRT =10 d) achieving steady-state for almost 2 months.

To increase the confidence of the results, a second independent reactor (MR2) was operated during 70 d (Fig. S1). The start-up period lasted 10 d with an HRT of 20-26 d and an OLR of 0.3-0.5 g COD/L d. Then, the HRT was switched to 10 d and the reactor was operating at an OLR of 1 g COD/L d for 1 month. To encourage the methanogenic activity the OLR was changed to 2 g COD/L d by increasing the COD concentration of the synthetic feeding and keeping the HRT at 10 d. In this case, steady-state operation was achieved for 1 month. The applied OLR values (1-2 g COD/L d) were selected to approach the typical operating range of industrial sewage sludge anaerobic digesters (Green and Perry, 1999).

Temperature and biogas production were monitored daily. The rest of conventional parameters (i.e., pH, alkalinity, VFA, COD, solid concentrations, and biogas composition) were analyzed twice a week to check the performance of both reactors.

2.3 OMPs fate through kinetic experiments

The fate of OMPs was evaluated via three experiments (Experiment I in MR1, days 119-128; Experiment II in MR2, days 27-34; and Experiment III in MR1, days 61-68) performed in continuous reactors under steady-state operation. Each kinetic experiment was initiated by adding a pulse of the selected OMPs to the corresponding MR to attain an initial in-reactor concentration of 10 µg/L for hormones (E1, E2, and EE2) and 100 µg/L for the other compounds. This methodology appears as more reliable since the microbiome has already been acclimated without substrate limiting conditions. The kinetics were directly performed inside the reactors, which avoids the risk of operating with non homogeneous inoculum, as it may happen in conventional batch experiments.

The added concentrations of OMPs are based on their occurrence in sewage sludge (Gonzalez-Gil et al., 2016), which can reach values above 100 µg/L for some compounds (i.e. musk fragrances). To provide a sound comparison of their biotransformation behavior, this concentration was used for all OMPs, except for hormones, which are usually detected below 10 µg/L in sewage sludge and higher concentrations could strongly modify the estrogenicity of the reactors. The concentrations in the liquid (8 time points) and solid phases (5-6 time points) of the effluent were monitored for 10 d. The first sample was withdrawn 10 min after the OMPs spike to allow for homogeneity and equilibrium between phases. All samples were immediately centrifuged at 3500 rpm for 30 min and OMPs were quantified in both liquid and solid phases (see section 2.5). During the kinetic experiments, the conventional parameters of the reactors were monitored daily.

2.4 Determination of sorption and biotransformation

The total concentration of the compounds (sum of the solid and liquid phase) was adjusted to pseudo-first order kinetics to determine the biotransformation rate constant, a widely accepted criterion to model OMPs biotransformation in biological processes (Pomiès et al., 2013). As the experiments were performed in continuous operation to keep the normal microbial activity, OMPs washout from the reactors was estimated (Equation 1) and included in the kinetic model (Equation 2). The pseudo-first order biotransformation rate constants (k_{biol}) were robustly estimated following a bootstrap procedure implemented in Matlab® (more details are included in section S2 of Supplementary data).

To quantify OMPs sorption, the *Kd* was estimated (Equation 3), since this linear isotherm is valid at trace concentrations and is widely used to describe the OMP distribution in biological systems (Carballa et al., 2008; Pomiès et al., 2013; Ternes et al., 2004).

$$C_{wash} = C_0 \cdot e^{(-t/HRT)} \qquad \textit{Equation 1}$$

$$C_T = C_0 \cdot e^{\left(-\frac{1}{HRT} - k_{biol} \cdot X_{VSS}\right) \cdot t} \qquad \textit{Equation 2}$$

$$Kd = \frac{C_s}{C_L \cdot X_{TSS}} \qquad \textit{Equation 3}$$

where C_{wash} is the concentration of an OMP (µg/L) remaining in the reactor if only washout took place (not undergoing any biological transformation); C_T is the total concentration of an OMP (µg/L); C_0 is the initial concentration of the compound (µg/L); C_s is the concentration in the solid phase (µg/L), C_L is the concentration in the liquid phase (µg/L); *t* is the time after the pulse of OMPs (d); *HRT* is the hydraulic retention time of the MR (d); k_{biol} is the pseudo-first order biotransformation rate constant (L/g VSS d); X_{VSS} is the concentration of volatile suspended solids (g/L); X_{TSS} is the concentration of total suspended solids (kg/L); *Kd* is the solid-liquid partition coefficient (L/kg).

Total biotransformation efficiency (B_T) was defined as the sum of the apparent biotransformation calculated in the liquid (B_L) and solid phase (B_S) (Equation 6). The depletion of the OMPs in each phase was estimated as the relative difference between the concentration if only washout (without biotransformation) took place and the measured experimental concentration. This term was weighted by the ratio between the measured concentration in each phase over the total concentration to obtain the corresponding apparent biotransformation (Equations 4-5), i.e., the contribution of each phase to the total

biotransformation. Apparent biotransformation efficiencies were calculated for each time point and the maximum value obtained after 4 days was selected since it remained almost constant for all of the OMPs. It should be noticed that the resulting biotransformation efficiencies refer to the corresponding C_{wash} instead of C_0.

$$B_L = \frac{C_L}{C_T} \cdot \frac{C_{wash\_L} - C_L}{C_{wash\_L}} \cdot 100 \qquad \textit{Equation 4}$$

$$B_S = \frac{C_S}{C_T} \cdot \frac{C_{wash\_S} - C_s}{C_{wash\_S}} \cdot 100 \qquad \textit{Equation 5}$$

$$B_T = B_L + B_S \qquad \textit{Equation 6}$$

2.5 Analytical techniques

2.5.1 Determination of physicochemical parameters

Total and partial alkalinity, pH, ammonium, total and soluble COD, total and volatile solids (TS, VS), and total and volatile suspended solids (TSS, VSS) were monitored according to standard methods (APHA, 2005). The concentration of VFA (acetic, propionic, butyric) was determined using a gas chromatograph (HP 5890A) with a Flame Ionization Detector (HP 7637A). Biogas production was measured continuously using a Ritter milligascounter (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany) and its composition was determined through gas chromatography (HP 5890 Series II).

2.5.2 Quantification of OMPs

The liquid phase of the reactor effluent was pre-filtered (AP4004705, Millipore) and filtered by 0.45 μm (HAWP04700, Millipore). Then, solid phase extraction (SPE) with 60 mg OASIS

HLB cartridges (Waters, Milford, MA, USA) was applied to 100 mL of the filtered sample (Gonzalez-Gil et al., 2016). Ultrasonic solvent extraction (USE) was used to quantify the OMPs sorbed onto the methanogenic sludge. Freeze-dried solids (approximately 0.5 g) were extracted five times with methanol and acetone. In each extraction, the samples were ultrasonicated for 15 min and then centrifuged at 1500 rpm for 5 min. All of the supernatants were combined, filtered through glass wool and evaporated (R-205, Büchi) under vacuum conditions at 40 ºC. The concentrated extract was diluted with distilled water, and SPE was conducted similar to the liquid samples but using 200 mg OASIS HLB cartridges (Waters, Milford, MA, USA).

Anti-inflammatories (IBP, NPX, DCF), several endocrine disrupting compounds (BPA, TCS, NP, OP), and musk fragrances (HHCB, AHTN, ADBI) were determined using a gas chromatograph (Varian CP-3900) coupled to an ion trap spectrophotometer (Varian CG-2100). The quantification of the neurodrugs (FLX, CBZ, DZP), antibiotics (ERY, ROX, SMX, TMP), and hormones (E1, E2, EE2) was performed using an Agilent G1312A liquid chromatograph with a binary pump and an HTC-PAI automatic injector (CTC Analytics) connected to a API 4000 triple quadrupole mass spectrometer (Applied Biosystems).

Limits of quantification (LOQ) of the SPE extracts were set as the lowest calibration point with a signal/noise ratio above 10. LOQ of the sample (Table S2) were calculated dividing the LOQ of the extract by the enrichment factor (concentration in extract compared to the source, $L_{liquid\_sample}/L_{extract}$ and $g_{solid\_sample}/L_{extract}$). Absolute recoveries in both phases (n=3) were obtained following the procedure of Gonzalez-Gil et al., (2016) and are specified in Table S2. Other validation parameters of the method as linearity of calibration curves (R^2 >

0.99) and precision determined by intra-day (RSD values within 0.6-5.8%, n=6) and inter-day (RSD values within 1.9-9.7%, n=5) analysis were found satisfactory.

3. RESULTS AND DISCUSSION

3.1 Methanogenic reactors performance

Table 1 shows the detailed performance of both reactors during the three OMPs kinetic experiments. Experiments I and II were carried out under similar conditions (i.e., OLR, solids content, biogas composition, and VFA methanization) in both MRs operating satisfactorily under steady-state methanogenic conditions. During Experiment III, the higher OLR clearly increased the methanogenic activity in MR2, but also the VFA concentrations, which led to a higher ratio of intermediate to total alkalinity (0.4-0.5). A methanization efficiency of 70-80% was achieved during the three experiments. No significant variations were observed in the pH in both MRs, which was maintained in the neutral range (7.5-8.0) throughout the whole operation. Further details of the MRs operation are provided in Fig. S1.

Table 1. Average operational and performance parameters of both methanogenic reactors (MR1, MR2) during the OMPs kinetic experiments.

	MR1	MR2	
	Experiment I	Experiment II	Experiment III
Period (d)	119-128	27-34	61-68
Temperature (ºC)	37.0 ± 0.5	37.0 ± 0.5	37.0 ± 0.5
HRT (d)	10	10	10
OLR (g COD/L d)	1.1 ± 0.1	0.94 ± 0.13	1.9 ± 0.2
$pH_{effluent}$	7.9 ± 0.2	7.5 ± 0.1	8.1 ± 0.2
$VFA_{influent}$ (g HAc_{eq}/L)	8.8 ± 0.1	7.4 ± 0.5	14.1 ± 1.9
Intermediate/total alkalinity	0.37 ± 0.06	0.20 ± 0.05	0.24 ± 0.06
Total alkalinity (g $CaCO_3$/L)	5.0 ± 0.4	2.3 ± 0.7	6.0 ± 1.0
$COD_{s\ influent}$ (g /L)	10.9 ± 0.8	9.4 ± 1.0	18.8 ± 2.3
COD removal (%)	71.1 ± 4.8	79.8 ± 5.4	77.4 ± 6.0
$TSS_{effluent}$ (g/L)	8.9 ± 1.0	8.7 ± 0.8	9.9 ± 0.6
$VSS_{effluent}$ (g/L)	2.2 ± 0.2	3.3 ± 0.5	2.6 ± 0.2
CH_4 production (g COD/L d)	0.79 ± 0.03	0.77 ± 0.05	1.5 ± 0.1

3.2 Sorption of OMPs onto methanogenic biomass

The sorption of OMPs onto methanogenic biomass was evaluated in each kinetic experiment and the average *Kd* (Equation 3) for each compound was determined (Fig. 1). It was observed that the ratio between the compound concentrations in solution and sorbed onto the solids remained constant throughout the kinetic experiment, indicating that the equilibrium was reached very quickly (within 10 minutes), as previously observed (Dionisi et al., 2006; Pomiès et al., 2013).

Based on their partitioning, the OMPs can be classified into three groups: Group 1 comprises the most hydrophilic compounds (2 > log *Kd*> 1; 50 > % sorbed > 10), ROX, CBZ, TMP,

NPX, DCF, ERY, SMX, and IBP; in the second group are BPA, DZP and the three hormones, which present a medium hydrophobicity ($2.5 \geq \log Kd \geq 2$; $75 \geq$ % sorbed ≥ 50); and finally, Group 3 comprises compounds highly hydrophobic ($\log Kd \geq 2.5$; % sorbed ≥ 75), such as the three musk fragrances, TCS, OP, NP, and FLX. The low standard deviations confirm that the phase distribution of OMPs was practically constant along the three kinetic experiments.

Fig. 1 also includes the *Kd* values for digested sludge reported in the literature (Carballa et al., 2007; Clara et al., 2011; Gonzalez-Gil et al., 2016; Ivashechkin et al., 2004; Narumiya et al., 2013). Although the lack of studies measuring the *Kd* in anaerobic sludge (Table S4) hinders a more complete and accurate comparison, it can be observed that sorption onto methanogenic biomass and onto digested sludge are similar, suggesting that the sorption mechanisms and the distribution between phases are analogous.

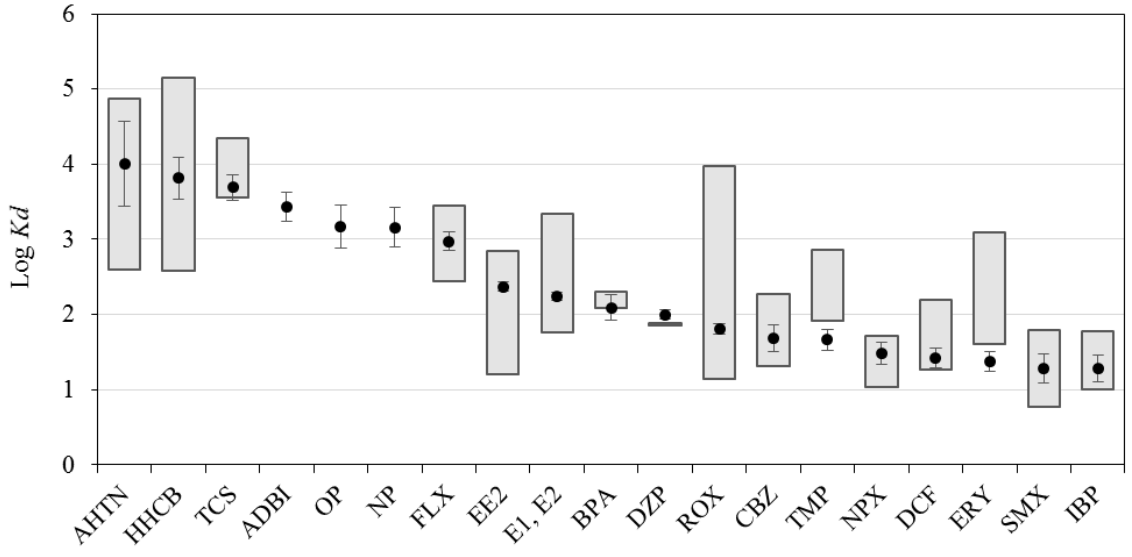

Fig. 1. Average partition coefficients (log *Kd*) of the 20 selected OMPs in methanogenic biomass (black points with standard deviation, n=16) and literature range (see detailed data in Table S4) for digested sludge (gray bars).

3.3 Biotransformation of OMPs under methanogenic conditions

The fate of OMPs during methanogenesis is depends heavily on their physicochemical properties. Fig. 2 shows the behavior of SMX, ERY, BPA, and FLX as representative compounds. The selection of these four compounds was based on the sorption (Fig. 1) and biotransformation (Table 2) behaviors observed during the kinetic experiments: i) highly biotransformed hydrophilic compounds (Group 1) (>70%) (SMX and TMP); ii) hydrophilic compounds (Group 1) with a lower than 60% biotransformation (NPX, ERY, CBZ, IBP, ROX, and DCF); iii) compounds with medium hydrophobicity (Group 2) and a biotransformation between 30-60% (EE2, BPA, DZP, and E1+E2); iv) hydrophobic compounds (Group 3) with a biotransformation between 40-70% (OP, NP, TCS, FLX, ADBI, HHCB, and AHTN). The data obtained for each compound in each kinetic experiment are reported in section S6 of Supplementary data.

As the experiments were conducted in continuous operation, there is a continuous OMP washout (solid line) from the reactor and the biotransformation was estimated as the difference between this virtual washout solid line and the measured concentration. Samples were analyzed in both solid and liquid phases to ascertain the role of biotransformation in each phase.

The average biotransformation efficiencies (Table 2) revealed that most OMPs reached a medium biotransformation level (35-70%) during methanogenesis. Only SMX and TMP showed higher efficiencies, while the biotransformation of CBZ, IBP, ROX, and DCF was below 35%. The comparison of these data with previous works is not straightforward as few studies have investigated the biotransformation of OMPs under methanogenic conditions and

available reported values correspond mostly to batch experiments. Nonetheless, previous studies revealed a high biotransformation for SMX (Cetecioglu et al., 2016), NPX (Lahti and Oikari, 2011), TCS (Veetil et al., 2012), and NP (Chang et al., 2005) while DCF was not biotransformed (Lahti and Oikari, 2011).

In an attempt to evaluate OMPs biotransformation kinetics, the overall (i.e., merging both the liquid and solid OMP concentration) biotransformation rate constant (k_{biol}) of each kinetic experiment was estimated via a pseudo-first order model (Equation 2), as shown in Table S5. The highest average k_{biol} values (>0.2 L/g VSS d) were obtained for NPX, SMX, and TMP; followed by OP, EE2, and NP (0.1-0.2 L/g VSS d). On the contrary, the lowest k_{biol} values (<0.025 L/g VSS d) correspond to CBZ, IBP, ROX, and DCF. For the rest of compounds, k_{biol} varied between 0.025 and 0.080 L/g VSS d. Reported biotransformation kinetic constants for anaerobic biomass are scarce, and no data were found in literature under methanogenic conditions. Higher k_{biol} values were obtained in batch anaerobic experiments for EE2 (0.9-1.5 L/g VSS d), NP (7.2 L/g VSS d), OP (2.2 L/g VSS d), BPA (0.5 L/g VSS d), and TMP (4.1 L/g VSS d) (Joss et al., 2004; Xue et al., 2010). In contrast, the results of Alvarino et al. (2014) (0.001-1.80 L/g VSS d) are in agreement with those of this study. Actually, they also found the highest biotransformation for NPX (k_{biol} = 1.1 L/g VSS d), SMX (k_{biol} = 0.36 L/g VSS d), and TMP (k_{biol} = 1.8 L/g VSS d).

Interestingly, the biotransformation rate generally slowed down or almost stopped after 2-4 d (Fig. 2 and Fig. S2) achieving a concentration plateau, especially in the case of hydrophobic compounds (Groups 2 and 3). This behavior cannot be described by pseudo-first order kinetics and therefore, the residuals between the measured and fitted concentrations are

relatively large (Table S5). A consequence of large fitting errors is the wide confidence interval of the biotransformation rate constant for some compounds (Table S5). This pattern was previously observed by Xue et al. (2010), who also reported large confidence intervals for first-order biotransformation rate constants under anaerobic conditions. Therefore, new modeling approaches that include some explanation for this fact, such as the bioavailability of the compound during AD (Delgadillo-Mirquez et al., 2011), should be evaluated to obtain more accurate biotransformation rate constants.

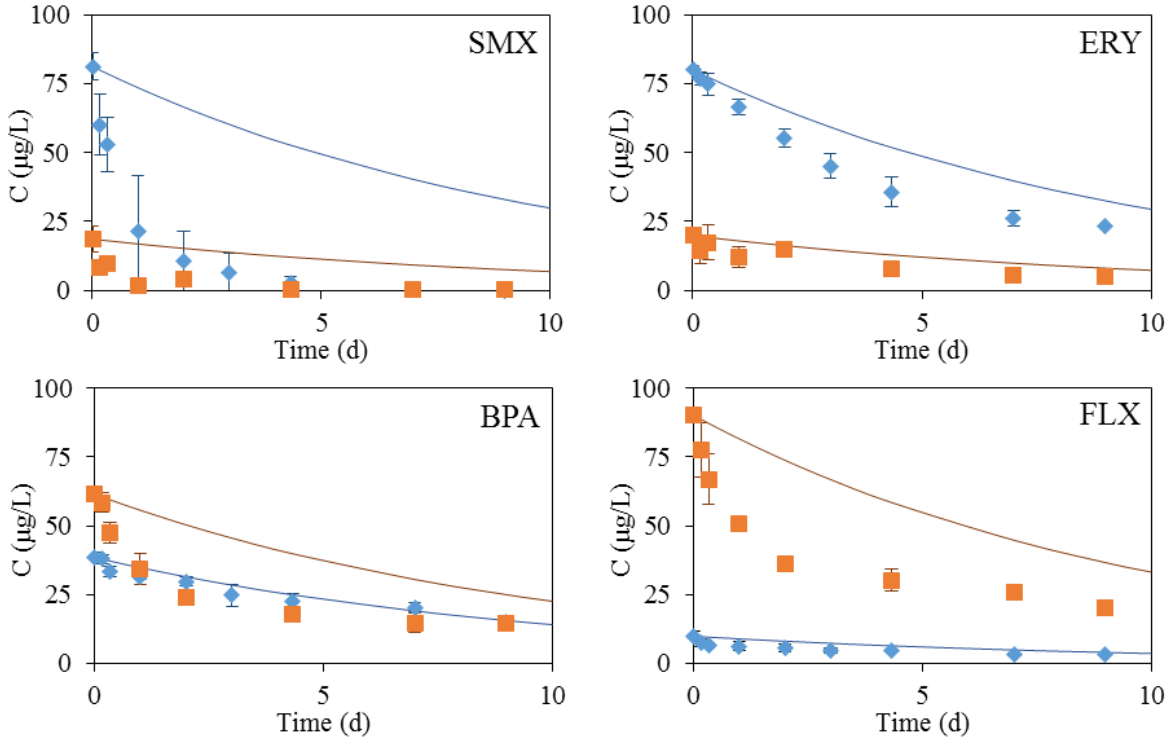

Fig. 2. Average fate of four representative OMPs during three kinetic experiments. Measured concentrations in the liquid (C_L, blue diamonds) and solid (C_s, orange squares) phases and estimated concentrations if only washout was responsible for their decrease in the liquid (C_{wash_L}) and solid (C_{wash_S}) phase (blue and orange continuous lines, respectively).

Table 2. Total biotransformation efficiencies (B_T) under methanogenic conditions and average contribution of the liquid (B_L) and solid phase (B_S). Mean values and standard deviations (SD) were obtained from the three experiments (n=3).

	B_T (%)				B_L (%)	B_S (%)
OMP	Experiment I	Experiment II	Experiment III	Mean ± SD	Mean ± SD	Mean ± SD
SMX	99	100	100	100±0	81±5	18±5
TMP	45	100	75	73±22	54±21	19±2
OP	72	61	n.a.	66±6	0±0	66±6
NPX	38	100	35	58±30	38±28	20±2
NP	69	42	n.a.	56±13	0±0	55±13
EE2	95	39	31	55±29	14±7	41±22
TCS	56	51	38	48±8	1±0	48±7
FLX	56	46	39	47±7	3±1	43±7
ADBI	46	41	49	45±3	3±1	42±4
HHCB	37	53	40	43±7	0±1	43±7
AHTN	46	50	33	43±7	2±0	41±8
BPA	27	45	44	39±8	4±3	35±5
ERY	30	45	35	37±6	29±6	8±2
DZP	37	47	23	36±10	18±7	17±3
CBZ	31	41	31	34±5	26±4	9±3
E1+E2	36	36	27	33±4	8±2	25±2
IBP	26	23	31	27±3	16±10	11±6
ROX	14	32	28	25±7	15±8	10±2
DCF	31	17	7	18±10	12±9	6±3

n.a. not available data

3.3.1 Influence of methanogenic activity

Several authors suggest that the cometabolic biotransformation of OMPs should improve when the overall metabolism of the microorganisms is promoted (Criddle, 1993; Delgadillo-Mirquez et al., 2011; Fernandez-Fontaina et al., 2014). Taking into account this hypothesis, the biotransformation of OMPs during Experiment III (OLR around 2 g COD/L d) should be

improved respect to Experiments I and II (OLR around 1 g COD/L d), since the methanogenic activity was almost doubled, in terms of CH_4 production (Table 1). However, when comparing results from experiments I and II to Experiment III (Table S5) no statistically significant differences were observed between OMPs biotransformation rates. In contrast, Alvarino et al. (2014) observed a linear relationship between the biotransformation rate (μg OMP/g VSS D) of SMX, TMP, and NPX and the methanogenic activity (40-140 mg COD/g VSS d) in a UASB reactor fed with skim milk diluted in water (1.5%, v/v). This discrepancy could be due to the magnitude of the methanogenic activity, being up to five times higher in our study (230-570 mg COD/g VSS d), which could be high enough to not limit the cometabolic transformation of OMPs.

The relationship between OMPs biotransformation and the microbial activity in AD process is still poorly investigated and controversial. Barret et al. (2010a) even observed a negative effect on the removal of some polycyclic aromatic hydrocarbons when the overall AD metabolism was enhanced, while most authors did not find a clear effect (Carballa et al., 2007; Gonzalez-Gil et al., 2016; Samaras et al., 2014; Yang et al., 2016). In this study, only NPX, SMX, TMP, EE2, and NP, presented significant differences ($\alpha = 0.05$) in the pseudo-first order biotransformation constant among the three experiments (Table S5). These differences were also reflected in the biotransformation efficiencies results (Table 2), except for SMX, which achieved a complete biotransformation in all cases. For TMP, NPX, and SMX the biotransformation was enhanced in Experiment II, while the biotransformation of EE2 and NP was higher in Experiment I.

3.3.2 Biotransformation phase and influence of the partition coefficient

The overall methanogenic biotransformation of OMPs is a consequence of its depletion in both the liquid and solid phase of sludge (Fig. 2). This aspect is further detailed in Table 2 and in Fig. S3, which highlights that the contribution of each phase to the overall biotransformation is closely related to the compound partitioning. Hydrophilic OMPs belonging to Group 1 (e.g. SMX and ERY) were mainly biotransformed in the liquid phase, while the contribution of the solid phase seemed more relevant for hydrophobic compounds (e.g. BPA of Group 2 and FLX of Group 3). These results do not allow identifying the phase where the biotransformation occurs, still a controversial topic in literature with hypotheses poorly justified experimentally (Pomiès et al., 2013).

The dissolved micropollutant is generally considered as the unique biodegradable fraction (Pomiès et al., 2013), because it is supposed that a sorbed compound is not available for microbial degradation (Delgadillo-Mirquez et al., 2011). However, some authors (Urase and Kikuta, 2005; Xue et al., 2010) assumed that biotransformation occurs only in the sludge phase after the compound is transferred from the liquid phase. The hypothesis of biotransformation occurring also in the sludge phase is supported by several reasons: i) sorption-desorption processes would limit the biotransformation of highly hydrophobic compounds (Fountoulakis et al., 2006); ii) the small size of most OMPs (<500 Da) allow them to diffuse through the outer membrane of bacteria (Nikaido and Vaara, 1985); iii) microorganisms are capable of degrading compounds directly from the sorbed phase during AD (Fountoulakis et al., 2006); iv) several extracellular (i.e. hydrolases) and intracellular enzymes (i.e. hydrolases, acetate kinase and monooxygenases) have been recently shown to

be able to biotransform OMPs in biological wastewater processes (Fernandez-Fontaina et al., 2016; Fischer and Majewsky, 2014; Gonzalez-Gil et al., 2017; Krah et al., 2016). Therefore, we hypothesize that OMPs can be biotransformed in both phases. Although the presence of extracellular enzymes in the MRs cannot be ruled out, methanogenesis is performed by intracellular enzymes (Christy et al., 2014; Gonzalez-Gil et al., 2017), so biotransformation inside the cell might be the main mechanism for most OMPs during methanogenesis.

Fig. 3 highlights that hydrophilic compounds (log *Kd* <2) presented high biotransformation variability (e.g. SMX vs. ERY, Groups 1), while the hydrophobic ones (log *Kd* > 3) remained in the medium range. Although some studies suggested that hydrophobicity would enhance the biotransformation of some OMPs (Wijekoon et al., 2015), it can be deduced from our data that the methanogenic biotransformation of the 20 selected OMPs is not influenced by their partitioning, agreeing with previously reported results for AD processes (Gonzalez-Gil et al., 2016; Yang et al., 2016).

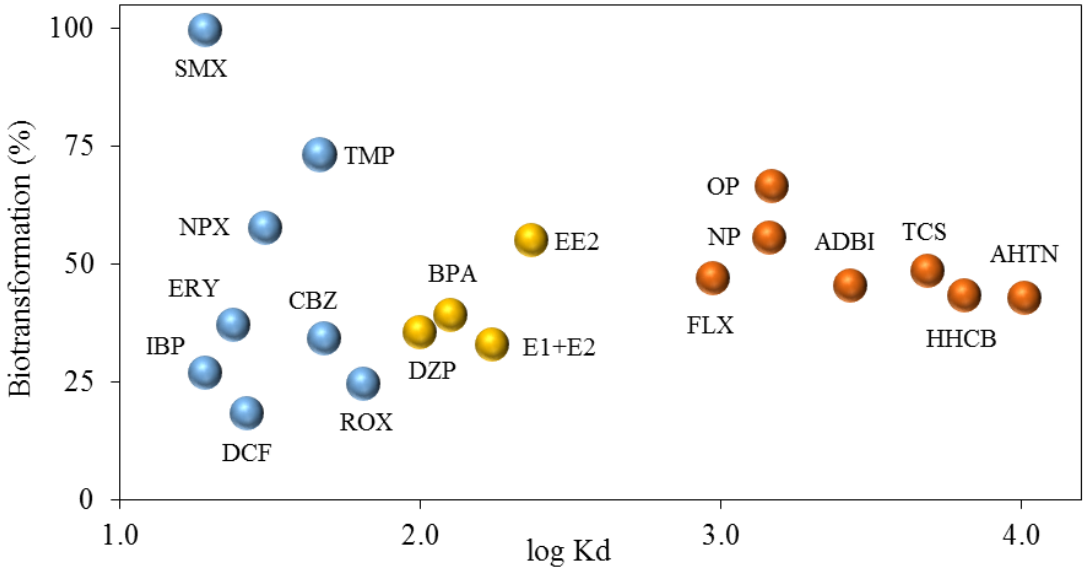

Fig. 3. Average biotransformation efficiencies and partition coefficients in methanogenic conditions. OMPs are classified into three groups based on their phase distribution (Group 1, blue; Group 2, yellow; Group 3, orange).

3.4 Methanogenic versus overall AD biotransformation

Fig. 4 compares the OMP biotransformation capacity of the methanogenic biomass versus the biotransformation efficiencies reported during AD of sewage sludge (Bergersen et al., 2012; Carballa et al., 2007; Clara et al., 2011; Gonzalez-Gil et al., 2016; Malmborg and Magnér, 2015; Narumiya et al., 2013; Paterakis et al., 2012; Samaras et al., 2014; Yang et al., 2016), which are summarized in Table S6. It should be pointed out that divergences in AD removal are common and that the reported data are still few, thus the relative differences between MR and AD (calculated as (AD – MR)/mean (AD, MR)) were only considered representative above 30%. According to this criterion, most OMPs (SMX, TMP, NP, EE2, TCS, ERY, BPA, DZP, and E1+E2) were biotransformed to a similar extent in the methanogenic step as in the global AD. Only NPX, IBP, ROX, and DCF presented a considerably lower biotransformation under methanogenic conditions, whereas FLX, the musk fragrances, and CBZ showed a higher biotransformation during methanogenesis than in AD.

Intuitively, it could be expected that hydrolysis would be the most efficient step to biotransform OMPs in AD, as a huge number of extracellular hydrolases are active in the liquid phase. However, our results suggest that the methanogenic step plays an important role in the overall removal of OMPs during AD. This conclusion agrees with Ghattas et al. (2017), who recently published a review on the anaerobic biotransformation reactions of emerging

organic contaminants in soil, sediment and wastewater treatment showing that a wide variety of bioreactions are conducted by methanogens.

Obviously, there could be also other communities involved in the anaerobic removal of OMPs, i.e. hydrolytic and acidogenic bacteria, which could lead to the higher values found for NPX, IBP, ROX, and DCF (Fig. 4). Nevertheless, even in those cases, the contribution of methanogenesis to the overall AD biotransformation is above 50%, except for ROX that is approximately one third. The higher biotransformation of FLX, musk fragrances and CBZ in the MR than in the AD could be explained by an increase in the bioavailability of the OMPs, as sorption occurs directly on the biomass responsible for biotransformation.

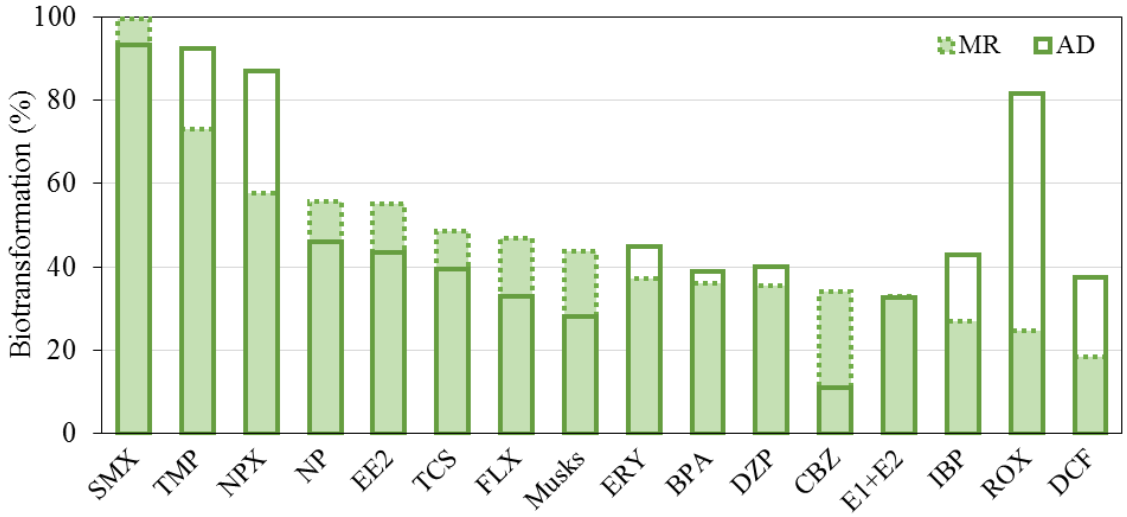

Fig. 4. Comparison between the average biotransformation efficiencies of OMPs in the methanogenic stage (MR) and in the overall AD process (see details of reported data in Table S6). Since HHCB, AHTN, and ADBI had similar biotransformation efficiencies, they are jointly represented as musks.

4. CONCLUSIONS

This study, performed with 20 OMPs presenting a wide variety of physicochemical and structural characteristics, demonstrates that methanogenesis is likely to play a major role in OMPs removal during AD. Biotransformation is not affected by the methanogenic activity in the range commonly found in sewage sludge digesters suggesting that this activity is enough to reach the maximum cometabolic biotransformation rate. It appears that biotransformation takes place in both the liquid and solid phase, as the partitioning of the compounds does not influence it. These results provide new insights into the anaerobic fate of OMPs; however, further research is needed to study the influence of other AD stages (e.g. hydrolysis) and to better understand the factors impeding a complete OMPs biotransformation.

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Supplementary data

Supplementary data related to this article can be found, in the online version, at

REFERENCES

Alvarino, T., Suarez, S., Lema, J.M., Omil, F., 2014. Understanding the removal mechanisms of PPCPs and the influence of main technological parameters in anaerobic UASB and aerobic CAS reactors. J. Hazard. Mater. 278, 506–513.

Angelidaki, I., Sanders, W., 2004. Assessment of the anaerobic biodegradability of macropollutants. Rev. Environ. Sci. Biotechnol. 3, 117–129.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Barret, M., Barcia, G.C., Guillon, A., Carrère, H., Patureau, D., 2010a. Influence of feed characteristics on the removal of micropollutants during the anaerobic digestion of contaminated sludge. J. Hazard. Mater. 181, 241–247.

Barret, M., Carrère, H., Latrille, E., Wisniewski, C., Patureau, D., 2010b. Micropollutant and sludge characterization for modeling sorption equilibria. Environ. Sci. Technol. 44, 1100–1106.

Bergersen, O., Østnes, K., Vasskog, T., 2012. Anaerobic treatment of sewage sludge containing selective serotonin reuptake inhibitors. Bioresour. Technol. 117, 325–332.

Carballa, M., Fink, G., Omil, F., Lema, J.M., Ternes, T., 2008. Determination of the solid-water distribution coefficient (Kd) for pharmaceuticals, estrogens and musk fragrances in digested sludge. Water Res. 42, 287–295.

Carballa, M., Omil, F., Ternes, T., Lema, J.M., 2007. Fate of pharmaceutical and personal care products (PPCPs) during anaerobic digestion of sewage sludge. Water Res. 41, 2139–50.

Cetecioglu, Z., Ince, B., Orhon, D., Ince, O., 2016. Anaerobic sulfamethoxazole degradation is driven by homoacetogenesis coupled with hydrogenotrophic methanogenesis. Water Res. 90, 79–89.

Chang, B. V., Chiang, F., Yuan, S.Y., 2005. Anaerobic degradation of nonylphenol in sludge. Chemosphere 59, 1415–1420.

Chen, F., Ying, G.-G., Ma, Y.-B., Chen, Z.-F., Lai, H.-J., Peng, F.-J., 2014. Field dissipation and risk assessment of typical personal care products TCC, TCS, AHTN and HHCB in biosolid-amended soils. Sci. Total Environ. 470–471, 1078–86.

Christy, P.M., Gopinath, L.R., Divya, D., 2014. A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. Renew.

Sustain. Energy Rev. 34, 167–173.

Clara, M., Gans, O., Windhofer, G., Krenn, U., Hartl, W., Braun, K., Scharf, S., Scheffknecht, C., 2011. Occurrence of polycyclic musks in wastewater and receiving water bodies and fate during wastewater treatment. Chemosphere 82, 1116–1123.

Criddle, C.S., 1993. The kinetics of cometabolism. Biotechnol. Bioeng. 41, 1048–1056.

Daughton, C.G., 2016. Pharmaceuticals and the Environment (PiE): Evolution and impact of the published literature revealed by bibliometric analysis. Sci. Total Environ. 562, 391–426.

Delgadillo-Mirquez, L., Lardon, L., Steyer, J.-P., Patureau, D., 2011. A new dynamic model for bioavailability and cometabolism of micropollutants during anaerobic digestion. Water Res. 45, 4511–21.

Dionisi, D., Bertin, L., Bornoroni, L., Capodicasa, S., Papini, M.P., Fava, F., 2006. Removal of organic xenobiotics in activated sludges under aerobic conditions and anaerobic digestion of the adsorbed species. J. Chem. Technol. Biotechnol. 81, 1496–1505.

Fernandez-Fontaina, E., Carballa, M., Omil, F., Lema, J.M., 2014. Modelling cometabolic biotransformation of organic micropollutants in nitrifying reactors. Water Res. 65, 371–383.

Fernandez-Fontaina, E., Gomes, I.B., Aga, D.S., Omil, F., Lema, J.M., Carballa, M., 2016. Biotransformation of pharmaceuticals under nitrification, nitratation and heterotrophic conditions. Sci. Total Environ. 541, 1439–1447.

Fischer, K., Majewsky, M., 2014. Cometabolic degradation of organic wastewater micropollutants by activated sludge and sludge-inherent microorganisms. Appl. Microbiol. Biotechnol. 98, 6583–6597.

Fountoulakis, M.S., Stamatelatou, K., Batstone, D.J., Lyberatos, G., 2006. Simulation of DEHP biodegradation and sorption during the anaerobic digestion of secondary sludge. Water Sci. Technol. 54, 119–128.

Ghattas, A.-K., Fischer, F., Wick, A., Ternes, T., 2017. Anaerobic biodegradation of (emerging) organic contaminants in the aquatic environment. Water Res. 116, 268–295.

Golet, E.M., Xifra, I., Siegrist, H., Alder, A.C., Giger, W., 2003. Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. Environ. Sci. Technol. 37, 3243–3249.

Gonzalez-Gil, L., Carballa, M., Lema, J.M., 2017. Cometabolic Enzymatic Transformation of Organic Micropollutants under Methanogenic Conditions. Environ. Sci. Technol. 51, 2963–2971.

Gonzalez-Gil, L., Papa, M., Feretti, D., Ceretti, E., Mazzoleni, G., Steimberg, N., Pedrazzani, R., Bertanza, G., Lema, J.M., Carballa, M., 2016. Is anaerobic digestion effective for the removal of organic micropollutants and biological activities from sewage sludge? Water Res. 102, 211–220.

Green, D.W., Perry, R.H., 1999. Perry's Chemical Engineers' Handbook, 7th ed. McGraw-Hill, New York.

Inglezakis, V., Zorpas, A.A., Karagiannidis, A., Samaras, P., Voukkali, I., Sklari, S., 2014. European Union legislation on sewage sludge management. Fresenius Environ. Bull. 23, 635–639.

Ivashechkin, P., Corvini, P.-X., Dohmann, M., 2004. Behaviour of endocrine disrupting chemicals during the treatment of municipal sewage sludge. Water Sci. Technol. 50, 133–140.

Joss, A., Andersen, H., Ternes, T., Richle, P.R., Siegrist, H., 2004. Removal of estrogens in municipal wastewater treatment under aerobic and anaerobic conditions: Consequences for plant optimization. Environ. Sci. Technol. 38, 3047–3055.

Krah, D., Ghattas, A.-K., Wick, A., Bröder, K., Ternes, T. a., 2016. Micropollutant degradation via extracted native enzymes from activated sludge. Water Res. 95, 348–360.

Lahti, M., Oikari, A., 2011. Microbial Transformation of Pharmaceuticals Naproxen, Bisoprolol, and Diclofenac in Aerobic and Anaerobic Environments. Arch. Environ. Contam. Toxicol. 61, 202–210.

Langdon, K.A., Warne, M.S.J., Smernik, R.J., Shareef, A., Kookana, R.S., 2011. Selected personal care products and endocrine disruptors in biosolids: an Australia-wide survey. Sci. Total Environ. 409, 1075–1081.

Lin, C.Y., Sato, K., Noike, T., Matsumoto, J., 1986. Methanogenic digestion using mixed substrate of acetic, propionic and butyric acids. Water Res. 20, 385–394.

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci. Total Environ. 473–474, 619–41.

Malmborg, J., Magnér, J., 2015. Pharmaceutical residues in sewage sludge: Effect of sanitization and anaerobic digestion. J. Environ. Manage. 153, 1–10.

Narumiya, M., Nakada, N., Yamashita, N., Tanaka, H., 2013. Phase distribution and removal of pharmaceuticals and personal care products during anaerobic sludge digestion. J. Hazard. Mater. 260, 305–12.

Nikaido, H., Vaara, M., 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49, 1–32.

Paterakis, N., Chiu, T.Y., Koh, Y.K.K., Lester, J.N., McAdam, E.J., Scrimshaw, M.D., Soares, A., Cartmell, E., 2012. The effectiveness of anaerobic digestion in removing estrogens and nonylphenol ethoxylates. J. Hazard. Mater. 199–200, 88–95.

Petrie, B., Barden, R., Kasprzyk-Hordern, B., 2015. A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. Water Res. 72, 3–27.

Plósz, B.G., Leknes, H., Thomas, K. V., 2010. Impacts of competitive inhibition, parent compound formation and partitioning behavior on the removal of antibiotics in municipal wastewater treatment. Environ. Sci. Technol. 44, 734–742.

Pomiès, M., Choubert, J.-M., Wisniewski, C., Coquery, M., 2013. Modelling of micropollutant removal in biological wastewater treatments: a review. Sci. Total Environ. 443, 733–48.

Qian, F., He, M., Song, Y., Tysklind, M., Wu, J., 2015. A bibliometric analysis of global research progress on pharmaceutical wastewater treatment during 1994–2013. Environ. Earth Sci. 73, 4995–5005.

Rubio-Loza, L.A., Noyola, A., 2010. Two-phase (acidogenic-methanogenic) anaerobic thermophilic/mesophilic digestion system for producing Class A biosolids from municipal sludge. Bioresour. Technol. 101, 576–585.

Samaras, V.G., Stasinakis, A.S., Thomaidis, N.S., Mamais, D., Lekkas, T.D., 2014. Fate of selected emerging micropollutants during mesophilic, thermophilic and temperature co-phased anaerobic digestion of sewage sludge. Bioresour. Technol. 162, 365–72.

Stasinakis, A.S., 2012. Review on the fate of emerging contaminants during sludge anaerobic digestion. Bioresour. Technol. 121, 432–40.

Ternes, T.A., Herrmann, N., Bonerz, M., Knacker, T., Siegrist, H., Joss, A., 2004. A rapid method to measure the solid-water distribution coefficient (Kd) for pharmaceuticals and musk fragrances in sewage sludge. Water Res. 38, 4075–4084.

Urase, T., Kikuta, T., 2005. Separate estimation of adsorption and degradation of pharmaceutical substances and estrogens in the activated sludge process. Water Res. 39, 1289–300.

Veetil, P.G.P., Nadaraja, A.V., Bhasi, A., Khan, S., Bhaskaran, K., 2012. Degradation of triclosan under aerobic, anoxic, and anaerobic conditions. Appl. Biochem. Biotechnol. 167, 1603–1612.

Wijekoon, K.C., McDonald, J.A., Khan, S.J., Hai, F.I., Price, W.E., Nghiem, L.D., 2015. Development of a predictive framework to assess the removal of trace organic chemicals by anaerobic membrane bioreactor. Bioresour. Technol. 189, 391–398.

Xue, W., Wu, C., Xiao, K., Huang, X., Zhou, H., Tsuno, H., Tanaka, H., 2010. Elimination and fate of selected micro-organic pollutants in a full-scale anaerobic/anoxic/aerobic process combined with membrane bioreactor for municipal wastewater reclamation. Water Res. 44, 5999–6010.

Yang, S., Hai, F.I., Price, W.E., McDonald, J., Khan, S.J., Nghiem, L.D., 2016. Occurrence of trace organic contaminants in wastewater sludge and their removals by anaerobic digestion. Bioresour. Technol. 210, 153–159.

Zhou, H., Zhang, Z., Wang, M., Hu, T., Wang, Z., 2017. Enhancement with physicochemical and biological treatments in the removal of pharmaceutically active compounds during sewage sludge anaerobic digestion processes. Chem. Eng. J. 316, 361–369.