Acidogenesis is a key step in the anaerobic biotransformation of organic micropollutants

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HIGHLIGHTS

- Acidogenesis and not hydrolysis sets the fate of OMPs during anaerobic digestion
- Biotransformation is the main mechanism for the acidogenic removal of OMPs
- Acidogenic enzymes and the molecular structure of OMPs drive biotransformation
- Acidogenic conditions favored the biotransformation of musks and macrolides

Acidogenesis is a key step in the anaerobic biotransformation of organic micropollutants

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Abstract: Understanding the role of the different anaerobic digestion stages on the removal of organic micropollutants (OMPs) is essential to mitigate their release from wastewater treatment plants. This study assessed the fate of 21 OMPs during hydrolysis and acidogenesis to elucidate the contribution of these stages to the overall anaerobic removal. Moreover, the removal mechanisms and factors influencing them were investigated. To this purpose, a fermentation reactor was operated and fed with two different substrates: starch (to jointly evaluate hydrolysis and acidogenesis) and glucose (to isolate acidogenesis). Results indicate that sulfamethoxazole was highly biotransformed (>80%), while galaxolide, celestolide, tonalide, erythromycin, roxithromycin, trimethoprim, octylphenol and nonylphenol achieved a 50-80% biotransformation. Since no significant differences in the biotransformation efficiencies were found between starch and glucose fermentation, it is stated that the enzymatic activities involved in starch hydrolysis do not significantly contribute to the cometabolic biotransformation of OMPs, while acidogenesis appears as the major player. Moreover, a higher biotransformation ($\geq$ 15 percentage points and $p \leq 0.05$) was found for galaxolide, celestolide, tonalide, erythromycin and roxithromycin during acidogenesis in comparison with the efficiencies reported for the acetogenic/methanogenic step. The biotransformation of some OMPs was explained considering their chemical structure and the enzymatic activities.

Keywords: Biodegradation, Hydrolysis, Fermentation, Methanogenesis, Partition coefficient.

1. INTRODUCTION

The presence of organic micropollutants (OMPs) in effluents from wastewater treatment plants (WWTPs) has been a growing concern as these compounds pose a threat to public health, biota and the economy, making drinking water treatment more expensive. These OMPs comprise pesticides, industrial chemicals, components of consumer products, pharmaceuticals, personal care products, hormones, and other organic pollutants that are ubiquitous in sewage and other environmental samples [1–3]. Their concentrations in the environmental matrices are quite variable, and the untreated wastewater concentrations (e.g. up to 2 $\mu g \cdot L^{-1}$ of sulfamethoxazole, 9 $\mu g \cdot L^{-1}$ of musks, 1 $\mu g \cdot L^{-1}$ of estrone and 5 $\mu g \cdot L^{-1}$ of triclosan) are usually smaller than those found in sewage sludge (e.g. up to 18 $\mu g \cdot L^{-1}$ of sulfamethoxazole, 141 $\mu g \cdot L^{-1}$ of musks, 8$\mu g \cdot L^{-1}$ of estrone and 38 $\mu g \cdot L^{-1}$ of triclosan) [4–6]. Understanding the distribution, fate, and behavior of these OMPs in biological treatments is a prerequisite to optimizing their elimination in WWTPs [7].

The main mechanisms involved in the biological removal of OMPs in WWTPs comprise sorption on the biomass and biotransformation through cometabolism [8–12]. Several factors may influence these mechanisms: physicochemical properties of each compound (hydrophobicity – octanol-water partitioning coefficient, charge – anionic or cationic form, and functional groups – presence of electron donating groups or withdrawing groups – EDGs or EWGs); operational conditions of the treatment system (sludge retention time, pH and temperature); redox conditions (anaerobic, anoxic and aerobic); diversification and adaptation of the microbial communities involved in the process; and presence of specific microbial enzymes [1,13–18]. For instance, recent studies have indicated that anaerobic systems can enhance the biodegradation of some OMPs, notably sulfamethoxazole, trimethoprim and naproxen [6,13,16,19–22], possibly

due to the negative redox potential of the environment and the presence of specific microorganisms and enzymes.

Anaerobic treatment is widely applied worldwide in several ways: as a main secondary treatment unit in tropical countries – e.g., UASB (Upflow Anaerobic Sludge Blanket) reactors [23]; digestion of primary and secondary sewage sludge; and as a preliminary unit in activated sludge plants that require a biological phosphorus removal [24]. Moreover, anaerobic conditions frequently occur in environmental matrices, such as soils, sediments, groundwater and landfills [25,26]. In all these natural environments and engineering applications, anaerobic digestion usually occurs through four main biological stages, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis, which are catalyzed by different microbial populations and enzymes. Many studies have reported removal efficiencies of OMPs in anaerobic systems [6,13,14,16,20,27–35], however research on the capacity of the four biological stages to degrade OMPs is still incipient, and most microorganisms, enzymatic processes, and anaerobic biotransformation pathways remain unknown [15]. A recent study performed by Gonzalez-Gil et al. [36] investigated the contribution of acetogenesis-methanogenesis to the OMP biotransformation during anaerobic digestion. However, the role of hydrolysis and acidogenesis is completely unexplored. It is expected that during these stages, several OMPs will be biotransformed, since hydrolytic microbes/enzymes are usually less specific and sensitive to the input of toxic xenobiotic compounds than methanogenic ones [37]. Furthermore, some authors [38–40] indicated that fermentative microorganisms are able to use organic compounds containing aromatic rings as electron acceptors (e.g. benzene, xylene, benzoate, phenol, and polycyclic aromatic hydrocarbons), and thus a similar behavior could be hypothesized for aromatic OMPs.

Actually, Duan et al. [41] recently proved that the surfactant nonylphenol is biotransformed under acidogenic stages.

The metabolic routes during hydrolysis and acidogenesis depend on the substrate composition. Carbohydrates are among the main constituents of various agroindustrial waste, municipal sewage and sewage sludge that are treated anaerobically [42,43]. Besides, carbohydrates are the preferred substrate for fermentative hydrogen-producing bacteria such as *Clostridium* species (sp.) [44] and they were more efficiently and rapidly degraded than proteins during sludge anaerobic digestion [45]. In addition, their metabolic routes are well known, and glycolysis is an obligatory route for the pyruvate formation with the sequential generation of soluble fermentation products [46].

In this context, the main objective of this study is to assess the role of (starch-)hydrolysis and (glucose-)acidogenesis on the anaerobic biotransformation of 21 OMPs. To this aim, the fate of OMPs in a fermentative reactor was evaluated; in particular, the mechanisms responsible for removing OMPs (i.e., biotransformation, sorption and abiotic reactions) and the main factors (e.g., chemical structure, partition coefficient and enzymatic activities) driving biotransformation were investigated. Finally, attempting to set the relevance of hydrolysis/acidogenesis versus acetogenesis/methanogenesis to the biotransformation of OMPs during the overall anaerobic digestion process, our experimental results were compared with bibliographic data from an acetogenic/methanogenic reactor [36].

2. MATERIALS AND METHODS

2.1. Hydrolytic/Acidogenic reactor

An anaerobic lab-scale (5 L) reactor was operated under mesophilic (37 °C) and acidic (pH of 5.0 ± 0.2) conditions. Based on the studies performed by Temudo et al. [46] and Regueira et al. [47], the highest biohydrogen (H_2) yields were reached at pH 5 during

glucose fermentation by mixed culture. The pH was controlled by NaOH (1.0 $mol \cdot L^{-1}$ - dosage of around 20 $mL \cdot d^{-1}$) and HCl (0.1 $mol \cdot L^{-1}$ - dosage of around 5 $mL \cdot d^{-1}$) dosing pumps linked to an online monitoring pH probe. The reactor was inoculated with biomass from a mesophilic sewage sludge digester and it was operated semi-continuously by once-a-day manual feeding and withdrawal of 1.25 L. To keep a high biomass concentration inside the reactor, a settling time of around 15 min was established before withdrawing the effluent. During the whole operation, the organic loading rate was kept at 3.1 g $COD \cdot L^{-1} \cdot d^{-1}$ [48] and the hydraulic retention time at 4 d [49]. The acidic pH hindered the methanogenic activity and no methane was detected in the biogas.

The reactor operation was divided into two periods depending on the carbon source. In the first period, a complex carbohydrate (i.e., soluble starch, Sigma-Aldrich) was selected to study the combined effect of hydrolysis and acidogenesis on the biotransformation of OMPs. In the second period, the main substrate was the monosaccharide glucose (Sigma-Aldrich) to avoid hydrolysis, and thus to specifically determine the contribution of acidogenesis. Apart from the carbon source (12.5 g $COD \cdot L^{-1}$ of starch or glucose) and the 21 OMPs, the synthetic feeding consisted of macro and micronutrients, based on Angelidaki and Sanders [50], as detailed in Table S1.

2.2. Selected organic micropollutants and sampling campaigns

The 21 OMPs selected for this study comprise four antibiotics: sulfamethoxazole (SMX), trimethoprim (TMP), erythromycin (ERY) and roxithromycin (ROX); three musk fragrances: galaxolide (HHCB), tonalide (AHTN) and celestolide (ADBI); three anti-inflammatories: ibuprofen (IBP), naproxen (NPX) and diclofenac (DCF); four

neurodrugs: fluoxetine (FLX), carbamazepine (CBZ), diazepam (DZP) and citalopram (CTL); four endocrine disrupting compounds from daily life products: bisphenol A (BPA), triclosan (TCS), 4-octylphenol (OP) and 4-nonylphenol (NP); and three estrogens: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2). The physicochemical characteristics of these compounds are very diverse, as shown in Table S2 of the supplementary material, and they are usually present in sewage sludge at varying concentrations. Therefore, based on the occurrence reported by Verlicchi et al. [51], Tran et al [5] and Clara et al. [52], the spiked concentrations were divided into four groups to obtain a realistic feeding mixture: 1 $\mu g \cdot L^{-1}$ of estrogens (E1, E2, EE2), 40 $\mu g \cdot L^{-1}$ of musk fragrances (HHCB, AHTN, ADBI), 20 $\mu g \cdot L^{-1}$ of endocrine disrupting compounds (BPA, TCS, OP, NP) and 10 $\mu g \cdot L^{-1}$ of the other OMPs (SMX, TMP, ERY, ROX, IBP, NPX, DCF, FLX, CBZ, DZP, CTL). Some recent studies [53,54] have pointed that, depending on the applied concentration, the OMPs could either promote or reduce the production of VFA. For instance, increasing the concentration of DCF from 2.5 $mg \cdot kg^{-1}$ to 25 $mg \cdot kg^{-1}$ had a positive effect on the reactor acidification performance, while higher doses of DCF (47 $mg \cdot kg^{-1}$TSS) decreased the VFA yield [53]. We did not expect a significant effect on the reactor performance at the low OMP concentrations added.

Six sampling campaigns were performed in the hydrolytic/acidogenic reactor to measure the OMP concentration in the feeding, the effluent, and the biomass: three campaigns (2 samples per campaign, n=6) during starch fermentation (hydrolysis + acidogenesis) and three campaigns (3 samples per campaign, n=9) during glucose fermentation (acidogenesis). All the samples were taken on different days, under steady-state conditions (Fig. 1).

2.3. Analytical methods

2.3.1. Conventional parameters

The reactor performance was monitored by physical-chemical analysis in influent, effluent and biomass samples of the reactor, according to the methods described in APHA [55]. The effluent (after biomass settling) and the biomass inside the reactor were analyzed twice a week by duplicate in terms of solids content (total solids (TS), total suspended solids (TSS) and volatile suspended solids (VSS)) and the total and soluble chemical oxygen demand (COD). Samples were taken from the effluent before biomass settling to analyze solids content and COD. The feeding was characterized in terms of COD. The acidification performance of the reactor was monitored twice a week by analyzing the concentration of volatile fatty acids (VFA) - acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, isocaproic and heptanoic - in the effluent using a gas chromatograph (HP 5890A) with a Flame Ionization Detector (GC/FID) (HP 7637A). Analysis of glucose, ethanol and formic acid were conducted by high performance liquid chromatography (Agilent 1100 series HPLC). The pH of the reactor was monitored continuously by a probe (CRISON PH 28). Biogas production was monitored via a Ritter milligascounter (Dr. Ing. Ritter Apparate- bau GmbH, Bochum, Germany) and samples from the headspace of the reactor were occasionally taken to analyze their composition through gas chromatography (HP 5890 Series II) equipped with a thermal conductivity detector (GC-TCD).

2.3.2. Organic micropollutants

The feeding and the effluent samples (250 mL) were pre-filtered (AP4004705, Milli-pore) and filtered by 0.45 mm (HAWP04700, Millipore) prior to performing solid phase extraction (SPE) with 60 mg OASIS HLB cartridges (Waters, Milford, MA, USA). The quantification of HHCB, AHTN, ADBI, IBP, NPX, DCF, TCS, NP, OP and BPA was

accomplished using a gas chromatograph (Varian CP-3900) coupled to an ion trap spectrophotometer (Varian CG-2100). The compounds ERY, ROX, SMX, TMP, FLX, CBZ, DZP, CTL, E1, E2 and EE2 were analyzed by a liquid chromatograph (Agilent G1312A) connected to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems). For the determination of OMPs in the solid phase, the biomass from the reactor was lyophilized to obtain approximately 0.5 g of TSS and subjected to an ultrasonic solvent extraction (USE) process, following a procedure based on Ternes et al. [56]. Three sequential extractions with methanol (4, 4 and 2 mL) and two with acetone (4 and 2 mL) were performed on the freeze-dried samples (0.5 g). After adding the corresponding solvent, samples were ultra-sonicated for 15 min and then centrifuged at 1500 rpm for 5 min. The resulting supernatants were combined (16 mL), filtered through glass wool, evaporated (TurboVap LV, Biotage) flowing nitrogen (200 kPa, 30 °C) and resuspended in 100 mL of Milli-Q water. Finally, SPE with 200 mg OASIS HLB cartridges and OMP quantification were performed as previously described for the liquid phase. Further details about the configuration of the instruments, parameter settings and limits of detection (LOD) and quantification (LOQ) for each OMP (Table S3) are provided in section S3 of the supplementary material.

2.4. COD mass balance and acidification of reactor

An organic mass balance (in g $COD \cdot d^{-1}$) in the reactor was performed (Eq. 1, based on Chernicharo [48]) in order to determine the mass distribution of the input COD that is used for biomass growth and converted into biohydrogen and soluble fermentation products, such as VFA, ethanol and formic acid. The biomass generated in the reactor by anabolism was considered equal to the biomass that is washed from the reactor, since the VSS content remains just about constant throughout each operational period. The

acidification efficiency (%) of the reactor can be calculated with Eq. 2 (based on Garcia-Aguirre et al. [57]).

$$Q_{inf} \,.\, COD_{inf} = Q_{eff} \,.\, (COD_{VFA} + COD_{SFP} + COD_{VSS}) + Q_{gas} COD_{H2} \quad (1)$$

$$Acidification\ (\%) = \frac{COD_{VFA}}{COD_{inf}} \cdot 100 \quad (2)$$

where Q_{inf}, $Q_{ef,}$ and Q_{gas} are the influent, effluent and output gas flows ($L \cdot d^{-1}$), respectively; COD_{inf} is the soluble COD measured in the feeding stream; COD_{VFA} represents the total equivalent COD of VFA measured in the effluent; COD_{SFP} refers to the COD equivalent in the effluent of other soluble fermentation products, e.g. ethanol and formic acid, calculated by the difference between the COD in the effluent and the COD_{VFA}; COD_{VSS} is the COD equivalent to the washout biomass, based on the experimental biomass growth coefficient ($Y_{biomass}$ – Table S4); and COD_{H2} represents the COD in the gaseous phase due to biohydrogen. Since H_2 was not measured, COD_{H2} was estimated by Eq. 1 and confirmed by theoretical calculations.

2.5. Micropollutant removal calculations

The relevant mechanisms involved in the total anaerobic elimination of OMPs comprise sorption and biotransformation. The total removal of each OMP was calculated by the difference in liquid phase concentration of the influent and effluent of the reactor, according to Yang et al. [18] and Phan et al. [16] (Eq. 3). Thus, to determine the biotransformation efficiency (Eq. 4), it has to be considered that part of the removed OMPs are leaving the reactor sorbed onto effluent solids (C_{sor}, Eq. 5). The contribution of sorption to the total removal (Eq. 3) was estimated with Eq. 6. Other parameters usually employed to quantify biotransformation and sorption were also calculated, namely, the pseudo-first order biodegradation constant (k_{biol}, Eq. 7 - according to Joss at

al. [58]) and the partition coefficient (K_D, Eq. 8 - according to Ternes et al. [59] and Carballa et al. [60]).

$$Total\ Removal\ (\%) = \frac{C_{inf} - C_{eff}}{C_{inf}} \cdot 100 = biotransformation\ (\%) + sorption\ (\%) \quad (3)$$

$$Biotransformation\ (\%) = \frac{C_{inf} - (C_{eff} + C_{sor})}{C_{inf}} \cdot 100 \quad (4)$$

$$C_{sor} = C_S \cdot TSS_{eff} \quad (5)$$

$$Sorption\ (\%) = \frac{C_{sor}}{C_{inf}} \cdot 100 \quad (6)$$

$$k_{biol} = \frac{ln\left(\frac{C_{inf}}{C_{eff}}\right)}{VSS \cdot HRT} \quad (7)$$

$$K_D = \frac{C_S \cdot 1000}{C_{eff}} \quad (8)$$

where C_{inf}, C_{eff}, C_{sor} is the concentration ($\mu g \cdot L^{-1}$) of the OMP in the influent, effluent (dissolved fraction) and sorbed onto effluent solids, respectively; TSS_{eff} is the total suspended solids in the effluent ($g \cdot L^{-1}$); C_S ($\mu g \cdot g^{-1}$TSS) is the concentration of OMPs in the solid phase; k_{biol} is the pseudo-first order biodegradation constant ($L \cdot g^{-1}VSS \cdot d^{-1}$); *VSS* is the concentration of volatile suspended solids in the reactor ($g \cdot L^{-1}$); and K_D is the partition coefficient ($L \cdot kg^{-1}$TSS).

2.6. pH influence on the OMP removal

Abiotic tests (without biomass) were carried out at pH 5 and pH 7 to prove the possible pH effect of the reactor on the elimination of the OMPs. The pH 5 was selected because it was the pH used in the operation of the hydrolytic/acidogenic reactor, while pH 7 is

248 the one usually applied in anaerobic digesters and in conventional biological treatment
249 systems [48,61]. In these assays, 3 flasks at each pH condition (5 and 7) were set up in a
250 buffer spiked with OMPs at the same concentrations as the reactor and kept stirred (150
251 rpm) at 37 °C for 72 h. Only the macrolides ERY and ROX showed a decrease in their
252 concentration at pH 5, which was not found at pH 7. Biotic assays (with acidogenic
253 biomass) were performed to corroborate and estimate the fraction of macrolides
254 biotransformed and abiotically removed by the pH effect in the reactor. 6 flasks were set
255 up with biomass from the acidogenic reactor (4.8 g $VSS \cdot L^{-1}$) and the synthetic medium
256 (described in Table S1) with 10 g $COD \cdot L^{-1}$ (as starch) spiked with 10 $\mu g \cdot L^{-1}$ of ERY,
257 ROX, and CBZ (used as no removal control). Then, the pH was adjusted for two
258 conditions – acid (pH 5, 3 flasks) and neutral (pH 7, 3 flasks). To maintain the pH
259 during the assay, $KHCO_3$ was used in a ratio of 1.2 g $KHCO_3 \cdot g^{-1}COD$. The flasks were
260 kept stirred (150 rpm) at 37 °C for 96 h. For inhibition of methanogenic archaea, 20
261 $mmol \cdot L^{-1}$ of BES (2-Bromoethanesulphonate) was added, according to Silva et al. [62].
262 The biogas composition in the headspace of the flasks was monitored once a day by gas
263 chromatography (HP 5890 Series II) equipped with a thermal conductivity detector
264 (GC-TCD), and the presence of methane was not detected. In both the abiotic and
265 abiotic assays, OMPs were analyzed at the initial time and after the incubation period,
266 following the procedure detailed in Section 2.3.2.

267 **2.7. Statistical Analysis**

268 Significant differences between the two operational phases of the reactor - starch and
269 glucose fermentation - were calculated by applying Analysis of Variance (ANOVA) of
270 one factor and Tukey's pairwise test. Likewise, the biotransformation efficiencies of
271 this study were statistically compared with those reported for methanogenesis and

overall anaerobic digestion. All of the statistical tests were performed at a 5% significance level by using the statistical software PAST 3.09 [63].

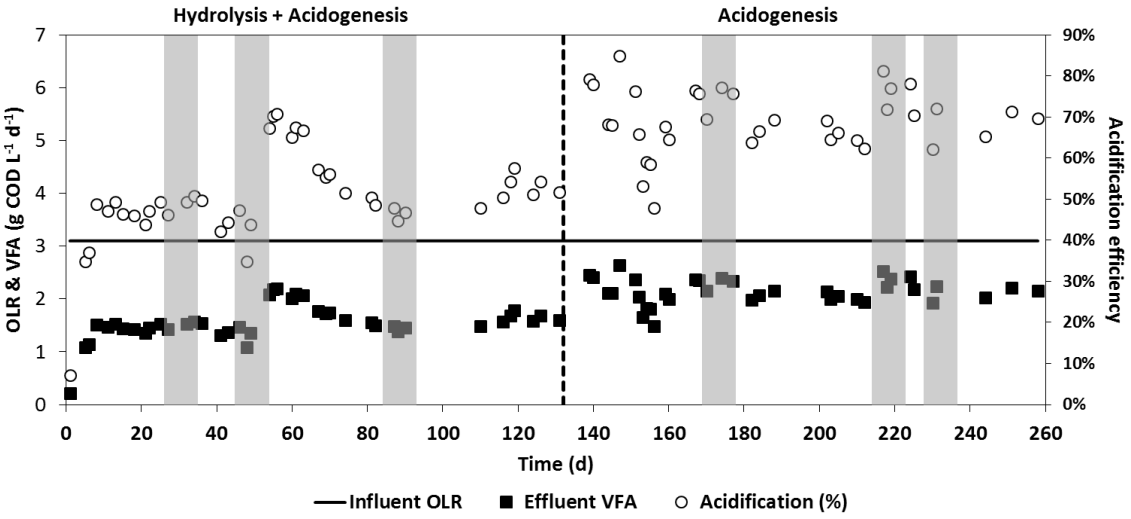
3. RESULTS AND DISCUSSION

3.1. Hydrolytic/Acidogenic reactor performance

The reactor was operated under steady-state conditions and presented good acidification performance (> 50%) throughout both operational periods (starch and glucose fermentation, Fig. 1). Further details of the reactor performance are summarized in Table S4 of the supplementary material. The substrate (starch or glucose) was not detected in the effluent, and approximately 6.5% was employed for biomass growth. Fig. S1 of the supplementary material shows the COD mass balance and average VFA composition and confirms that most of the substrate has been converted to VFA, and only a small fraction (10-20%) of the effluent COD corresponds to other soluble fermentation products. Formic acid (0.3%) and ethanol (4.1%) were detected during the glucose fermentation period. Since CH_4 was not detected in the biogas, the remaining COD might correspond to H_2. The estimated H_2 yield (Table S4) during starch fermentation was 2.7 mol $H_2 \cdot mol^{-1}$starch, whereas the values reported in the literature are in the range of 0.6 to 3.2 mol $H_2 \cdot mol^{-1}$starch [64–66]. During glucose fermentation, the H_2 yield (1.7 mol $H_2 \cdot mol^{-1}$glucose) was close to that obtained by Fang and Liu [67] (2.1 mol $H_2 \cdot mol^{-1}$glucose) and Lin and Chang [68] (1.7 mol $H_2 \cdot mol^{-1}$glucose), which also worked with mixed culture in an acidogenic reactor fed with glucose at pH around 5.6. The H_2 yields obtained from the COD mass balance (Eq. 1, Table S4) are comparable to the theoretically predicted H_2 yields (1.8 mol $H_2 \cdot mol^{-1}$starch and 2.3 mol $H_2 \cdot mol^{-1}$glucose) considering the stoichiometry of the metabolic network and the product spectrum of the reactor [47]. However, it cannot be excluded that part of the

297 starch is precipitated and not hydrolyzed, which in the COD mass balance would result
298 in an overestimation of the H_2 produced (Fig. S1).

299 It should be highlighted that the change of starch by glucose as carbon source led to an
300 increase in the acidification efficiency (from 52% to 69%) and a higher conversion into
301 butyric acid (Fig. S2 - temporal profile of VFA generation). The readily bioavailable
302 glucose concentration is much higher when it is directly fed to the reactor than when it
303 is limited by starch hydrolysis, which could result in a different metabolic pathway [69]
304 and a larger acidification efficiency. It could also be hypothesized that incomplete
305 starch hydrolysis to non-hydrolyzed intermediate polysaccharides could explain the
306 lower acidification efficiency during starch fermentation.



307

308 **Fig. 1.** Acidification performance of the reactor during hydrolysis + acidogenesis
309 (starch fermentation) and acidogenesis (glucose fermentation). Grey areas highlight the
310 periods when the OMPs sampling campaigns were conducted.

311

312 **3.2. Fate of OMPs during hydrolysis/acidogenesis**

313 There are three removal mechanisms influencing the fate of OMPs in the
314 hydrolytic/acidogenic reactor: biotransformation, sorption, and abiotic removal. In

Table S5 of the supplementary material, the biotransformation and sorption results of each operational period (starch and glucose fermentation) are shown. As no significant differences ($p > 0.05$, and differences below 10 percentage points) were found between both periods, the average contribution of each mechanism to the OMP removal throughout the reactor operation is plotted in Fig. 2. It can be noted that biotransformation is the main mechanism explaining more than half of the total removal observed for all the compounds, except for TCS that was mainly removed by sorption. Sorption was also relevant (> 15%) in the case of the three musk fragrances (HHCB, AHTN, and ADBI), OP, NP, and FLX. Finally, the abiotic removal at pH 5 was only significantly observed for ERY (40%). These three removal mechanisms are further discussed in the next sections.

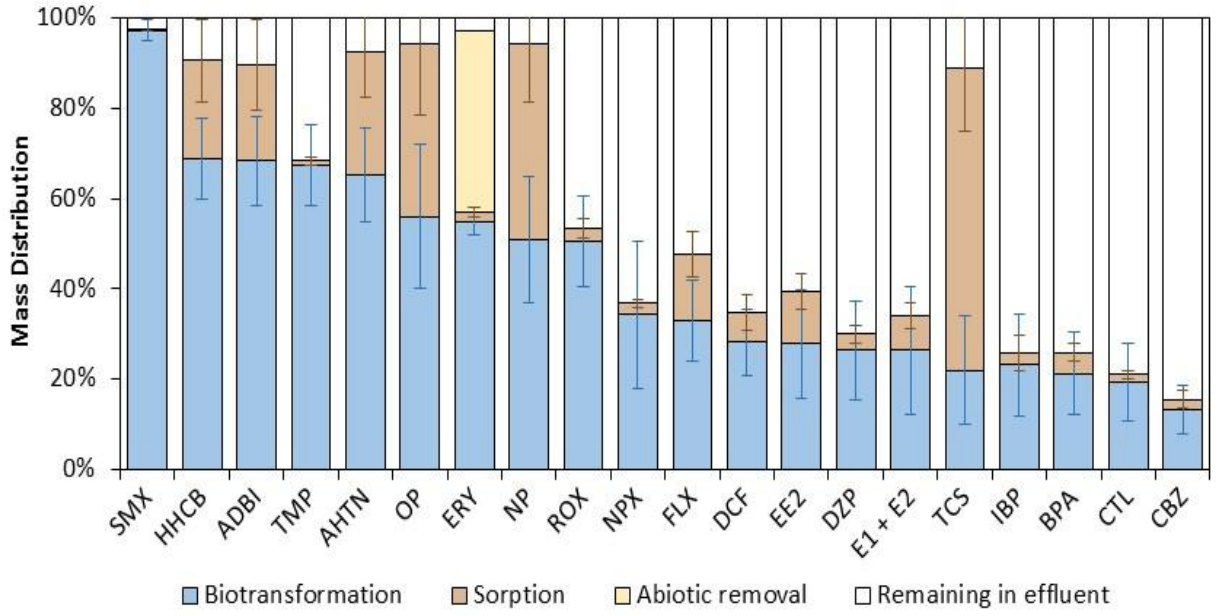


Fig. 2. Fate of OMPs in terms of biotransformation, sorption onto biomass, abiotic elimination and remaining fraction in the effluent. Average values and standard deviations throughout the reactor operation (n=15).

3.2.1 Abiotic elimination by pH effect

Fig. S3 presents the pH effect on the elimination of the macrolides ERY and ROX in the biotic and abiotic batch assays. It can be observed that ERY was almost completely removed (95%) at pH 5 in the abiotic and biotic assays. This behavior agrees with the results of Gobel et al. [70] and Atkins et al [71], who pointed out the chemical instability of ERY under acidic conditions and the formation of the inactive ERY-H_2O. However, in the hydrolytic/acidogenic reactor, biotransformation might occur concomitantly to the abiotic removal. Indeed, when the pH was set to 7 no abiotic removal was observed and ERY was biotransformed to 55% in the biotic assays. Since the acidification performance of the biotic assays was similar at pH 5 and 7 (52 and 61%, respectively), it is considered that the biomass from the reactor has the same biotransformation capacity at both pHs. Therefore, it is assumed that 55% of ERY could be biotransformed and the remaining 40% abiotically removed in the hydrolytic/acidogenic reactor.

Regarding ROX, although a slight concentration decrease (15-20%) was observed at pH 5 in the abiotic and biotic assays, it was not significant ($p > 0.05$). Actually, despite ROX and ERY having a similar structure, ROX is more stable under acidic conditions, since this macrolide was specifically synthesized from ERY to avoid its decomposition in the gastric juice [72,73]. As expected, CBZ (negative control) proved to be highly recalcitrant in abiotic and biotic assays and not influenced by the pH.

3.2.2 Sorption of OMPs

Sorption of OMPs on biomass can be predicted according to the hydrophobicity of the compound [14,18,74], which is estimated by the octanol-water partition coefficient (K_{ow}) (the calculation of the apparent octanol-water partitioning coefficient at pH 5, D_{ow}, is detailed in Section S2). Actually, a relationship between the measured sorbed fraction (%) and the hydrophobicity of each OMP was found, except for FLX, as shown

in Fig. S4 of the supplementary material. Nevertheless, sorption is governed not only by hydrophobicity but also by electrostatic interactions with the sludge [5,16,34], cationic exchanges, cationic bridges, surface complexation and hydrogen bonding [75]. This explains the sorption behavior of FLX, a hydrophilic compound according to its octanol-water partition coefficient but positively ionized at pH 5 (it has basic moieties and a pKa of 9.8 – Table S2), and thus it presented a high sorption potential due to electrostatic interaction with the sludge (negatively-charged) [76]. Since the partition coefficient (K_D, Eq. 8) covers all these phenomena, it is considered the best parameter to quantify the solid-liquid distribution of OMPs. According to their K_D values (Fig. 3), the OMPs were classified into three groups [36]: high sorption ($\log K_D \geq 2.5$), moderate sorption ($2.0 < \log K_D \leq 2.5$); low sorption ($\log K_D \leq 2.0$). Moreover, in Fig. 3 the average K_D values obtained in the hydrolytic/acidogenic reactor are compared with the literature range of anaerobic processes (data detailed in Table S6 of supplementary material), revealing a similar sorption behavior.

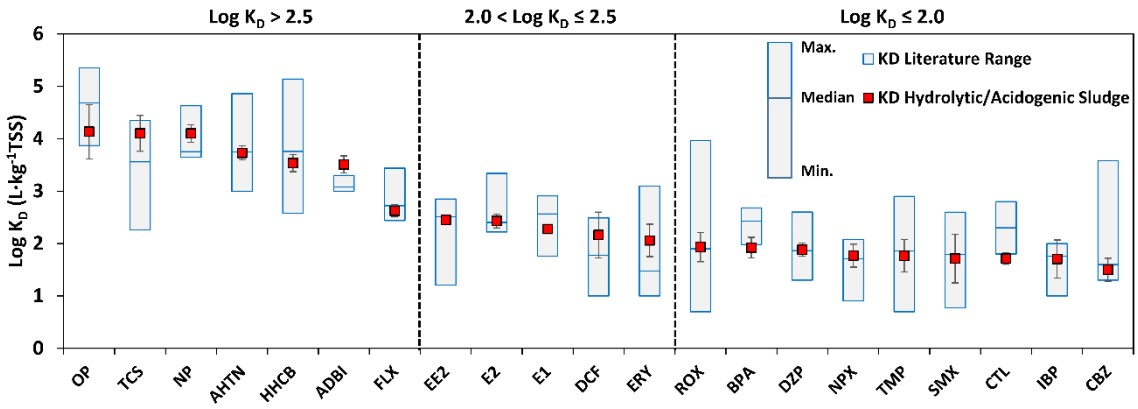


Fig. 3. Comparison between the partition coefficient ($\log K_D$ - $L \cdot kg^{-1}TSS$) in the hydrolytic/acidogenic biomass (n=15) and the literature range for anaerobic sludge (data detailed in Table S6).

3.2.3 Biotransformation

The biotransformation efficiencies (%) and kinetics (k_{biol}) attained in the hydrolytic/acidogenic reactor are presented and compared with the bibliographic results in Fig. 4 and Table S7 of supplementary material, respectively. The OMPs were classified according to their biotransformation in: (*i*) compounds highly biotransformed ($\geq$ 80% and $k_{biol} \geq 0.1$ $L \cdot g^{-1}VSS \cdot d^{-1}$) – SMX; (*ii*) compounds with a medium biotransformation (50-80% and $0.04 < k_{biol} < 0.1$ $L \cdot g^{-1}VSS \cdot d^{-1}$) – TMP, ADBI, HHCB, AHTN, OP, NP, ROX and ERY; (*iii*) and OMPs with a moderate-low biotransformation ($\leq$ 50% and $k_{biol} \leq 0.04$ $L \cdot g^{-1}VSS \cdot d^{-1}$) – NPX, DCF, DZP, TCS, FLX, EE2, IBP, E1 + E2, CTL, BPA and CBZ.

To further understand why different OMPs have different biotransformation efficiencies, we have to consider the molecular structure of the compounds (Table S2) that may exert a great influence. According to Wijekoon et al. [14], compounds containing only EDGs (e.g., $-NH_2$; $-NHR$; $-NR_2$; $-OH$; $-CH_3$; $-OCH_3$; $R-CO-R$) could be more susceptible to be anaerobically biotransformed, while those with EWGs (e.g., $-C\equiv N$; $-CF_3$; $-COOH$; $-COOR$; $-COR$; $-Cl$; $-F$; $-CONH_2$; $-CHO$) could be more recalcitrant. For instance, the low biotransformation of CBZ observed in the hydrolytic/acidogenic reactor is in agreement with literature data (Fig. 4, Table S8a), and it shows that the presence of the EWG ($-CONH_2$) might explain its recalcitrant behavior. In the same way, the presence of EWGs in the hydrophilic compounds IBP ($-$ COOH), DCF ($-$COOH; $-$Cl), CTL ($-$F, $-C\equiv N$) and DZP ($-$Cl), and in the hydrophobic compounds TCS ($-$Cl) and FLX ($-CF_3$,$-$COR) may be a key factor for its low biotransformation (Fig. 4). On the other hand, the complete (or almost complete) biotransformation of SMX in the hydrolytic/acidogenic reactor and in different anaerobic systems (Fig. 4, Table S8a) could be attributed to the presence of EDGs in its molecular structure ($-NH_2$, $-CH_3$, $-$NHR). Likewise, the presence of a hydroxyl bond

401 (EDG) seems to favor the anaerobic biotransformation of the hydrophobic surfactants
402 OP and NP. In contrast, the presence of this EDG in the E1, E2, and EE2 molecules did
403 not lead to relevant biotransformation efficiencies (Fig. 4).

404 Although the presence of EDGs and EWGs is an important factor in the OMP
405 biotransformation, it may be also influenced by other features related to the
406 bioavailability and physicochemical characteristics of the compounds such as
407 hydrophobicity, partition coefficient and molecular weight [14,35,77]. For instance, the
408 compounds with higher sorption capacity (log K_D > 2.5) and with EDGs in their
409 molecular structure - AHTN, HHCB, ADBI, OP, NP, showed a medium
410 biotransformation efficiency (50 - 80%), suggesting that biotransformation might occur
411 after sorption onto the biomass. Indeed, compounds with EDGs but with lower sorption
412 capacity (log K_D < 2.5 - E1/E2, EE2, and BPA) were poorly biotransformed (Fig. 4).

413 In order to evaluate the relevance of hydrolysis/acidogenesis on the biotransformation
414 of the OMPs during anaerobic processes, our k_{biol} values were compared with those
415 previously obtained by Alvarino et al. [19] in a pilot-scale UASB reactor. Despite most
416 compounds presenting similar k_{biol} values in both systems (Table S7), some OMPs
417 changed their biotransformation category: TMP and NPX with a moderate and low
418 biotransformation during hydrolysis/acidogenesis presented a higher rate in the UASB
419 reactor, while the k_{biol} of HHCB, AHTN, ROX, and ERY was medium in the
420 hydrolytic/acidogenic reactor and low in the UASB reactor. Unfortunately, few
421 anaerobic k_{biol} data are available in the literature, and thus a broader comparison is
422 established in Fig. 4 with the removal efficiencies reported for a variety of anaerobic
423 systems at bench, pilot and full scale (i.e., batch experiments, sludge digesters, UASB
424 reactors, and anaerobic membrane bioreactors). The details about the removal
425 efficiencies and the operational parameters of each study are provided in Tables

S7a/S7b. As can be inferred from Table S8 and according to many authors [6,18,28,33,34,78], no clear correlations were found between operating conditions and OMP biotransformation, which is why data obtained in different anaerobic technological applications were used to compare our k_{biol} values (Table S7) and biotransformation efficiencies (Fig. 4). It should be noted that the comparison of Fig. 4 is qualitative, since most references do not separate the total removal (biotransformation + sorption) from what is actually biotransformed. In addition, for some compounds, the reported results either show a large deviation or are scarce ($n \leq 5$ for ADBI, AHTN, OP, ERY, and CTL). Nonetheless, it could be inferred from Fig. 4 that the anaerobic biotransformation of most OMPs might cometabolically occur during the hydrolysis/acidogenesis of carbohydrates; even for some compounds (i.e., HHCB, ADBI, and ERY) it appears to be favored during this anaerobic stage, as previously observed with k_{biol} data. Only for some OMPs (i.e., TMP, NPX, and CTL), the hydrolytic/acidogenic step seems to play a minor role in their anaerobic removal, thus other anaerobic stages as acetogenesis, methanogenesis or even hydrolysis/acidogenesis of other substrates (e.g., proteins and lipids) might be responsible for their biotransformation.

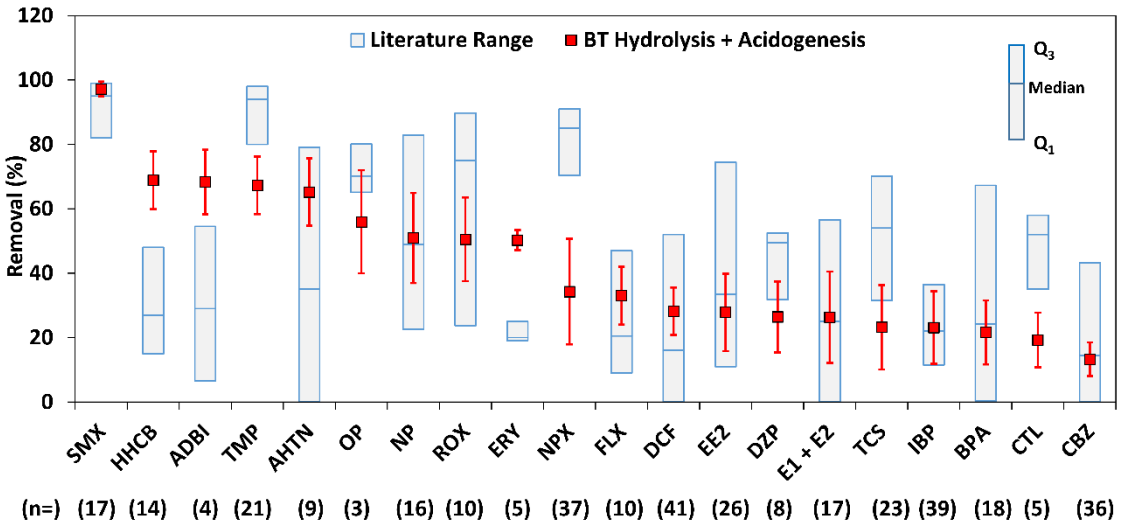


Fig. 4. Comparison between the OMPs biotransformation (BT) in the

hydrolytic/acidogenic step (n=15) and the reported removal values of the overall anaerobic digestion process. The literature data range is detailed in Table S8a. The number of reported values is shown in brackets (n=). Since E1 is converted naturally into E2 under anaerobic conditions [79], the results in terms of biotransformation are computed together (E1 + E2).

3.3. Anaerobic stages involved in the biotransformation of OMPs

3.3.1 Hydrolysis versus acidogenesis

Starch and glucose fermentation are similar processes since starch consists of a large number of glucose units joined by glycosidic bonds, and therefore they only differ in the first step, starch hydrolysis to glucose, which is mainly conducted by glycosidase enzymes (Fig. S5 - enzymatic pathway of starch hydrolysis to glucose). Then, acidogenic enzymes degrade glucose into VFA and other soluble fermentation products. The acidogenic fermentation of glucose represents a key step during anaerobic degradation of the wide variety of industrial wastes, as well as sewage and sludge from wastewater treatment plants [46,68,80,81].

We hypothesized that the higher activity of hydrolases (i.e., glycosidases) during starch fermentation might have resulted in a greater cometabolic biotransformation of certain OMPs (e.g., ERY and ROX that have a glycosidic bond). However, our experimental results do not support this theory. As shown in Fig. 5, all OMPs were biotransformed to the same extent in both operational periods: starch (hydrolysis + acidogenesis) and glucose (acidogenesis) fermentation. Actually, the results were statistically equal (ANOVA with a 5% significance level) and the differences between both biotransformation efficiencies did not exceed 10 percentage points for any OMP. These results suggest that hydrolysis of carbohydrates plays a minor role in the anaerobic

biotransformation of OMPs, while acidogenesis should be considered as a key step. It cannot be ruled out that, apart from glycosidases, other hydrolases might be present during starch and even glucose fermentation. However, their activity and cometabolic action towards the OMPs may be low in comparison with the enzymes involved in the metabolism of the main carbon source (starch or glucose).

Finally, it should be noted that the differences found in the performance of both operational periods (starch and glucose fermentation), particularly butyric acid conversion (see Section 3.1) did not affect the biotransformation of OMPs. Thus, either these differences did not alter the metabolic pathways for glucose acidification or the different pathways presented common enzymatic activities responsible for the biotransformation of OMPs.

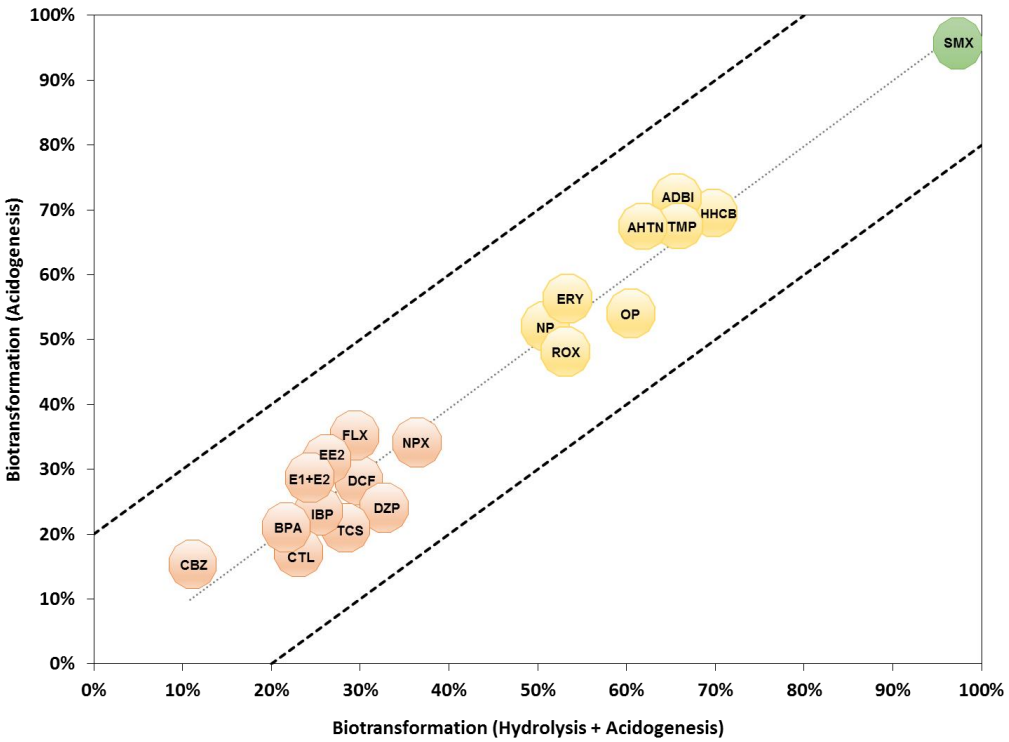


Fig. 5. Correlation between the OMPs biotransformation in hydrolysis + acidogenesis (starch fermentation, n = 6) and only the acidogenic step (glucose fermentation, n = 9). The detailed values are summarized in Table S5.

3.3.2 Acidogenesis versus methanogenesis

In Fig. 6, the biotransformation efficiencies attained in the fermentation reactor are compared with those obtained by Gonzalez-Gil et al. [36] in a methanogenic reactor fed with a mixture of VFA and enriched in heteroacetogens and aceticlastic methanogens. Overall, it can be concluded that acidogenesis and acetogenesis/methanogenesis are involved in the anaerobic biotransformation of the studied OMPs. However, the relative contribution of each stage differs among compounds, which can be divided into three groups (Fig. 6): *(a)* OMPs with a biotransformation significantly ($p \leq 0.05$) higher in acidogenic conditions (HHCB, ADBI, AHTN, ERY, and ROX); *(b)* compounds equally biotransformed in both stages ($p > 0.05$); and *(c)* OMPs whose biotransformation is significantly ($p \leq 0.05$) favored in acetogenic/methanogenic conditions (FLX, CBZ, BPA, and TCS).

As shown in Fig. 6, no significant differences ($p > 0.05$) between the biotransformation efficiencies during acidogenesis and acetogenesis/methanogenesis were observed for most OMPs (group *b*), indicating that both microbial populations have the same capacity to transform them despite having very different metabolic pathways. Indeed, SMX, which is completely biotransformed in acidogenesis and acetogenesis/methanogenesis, possibly undergoes a cleavage of its isoxazole ring under iron-reducing conditions [82]. This reaction might be indirectly mediated by the action of cytochrome c or membrane-bound hydrogenases [83], involved in several biological pathways like aceticlastic methanogenesis and glucose catabolism [84]. Yet, for most

508 OMPs, it remains unknown if they follow the same or different biotransformation
509 pathways (catalyzed by common or specific enzymatic activities of each stage). In
510 agreement with our results, Braun et al. [85] did not find significant differences between
511 the removal of polycyclic aromatic hydrocarbons in anaerobic reactors with
512 fermentative, methanogenic and intermediate activities, suggesting that
513 biotransformation of these compounds does not depend on the main metabolic activities
514 or the dominant microbial communities of the reactor.

515 On the other hand, OMPs from groups *(a)* and *(c)* presented differences in the
516 biotransformation capacity of both stages, which indicates that the stimulation of
517 specific enzymatic activities in the fermentation or methanogenic reactors may have
518 contributed to the greater biotransformation. For instance, in the case of the macrolides
519 ERY and ROX, the higher biotransformation in acidogenesis could be due to the higher
520 activity of glycosylase enzymes, which could cleave their hexose sugar (cladinose).
521 Actually, this hypothesis is supported by experiments recently performed with
522 anaerobic sludge enzymes [83]. Regarding ADBI and AHTN, the dominance of
523 fermentative species during acidogenesis might have favored some enzymatic
524 mechanisms related to the biotransformation of these compounds, as it could be the case
525 of the enzyme 3-hydroxybutyryl-CoA dehydrogenase that reduces ketone groups to
526 convert acetoacetyl-CoA to 3-hydroxybutyryl-CoA in the butyric acid metabolic route.
527 Certainly, the greater activity of other acidogenic enzymes in the fermentation reactor
528 might also explain why compounds from the group *(a)* achieved a higher
529 biotransformation respect to the overall anaerobic digestion process (Fig. 4). On the
530 contrary, the biotransformation of BPA and TCS (group *(c)*, Fig. 6) was favored during
531 acetogenesis/methanogenesis, which could be due to an increase in the activity of

acetate kinase, a methanogenic enzyme able to phosphorylate their hydroxyl groups [17,86].

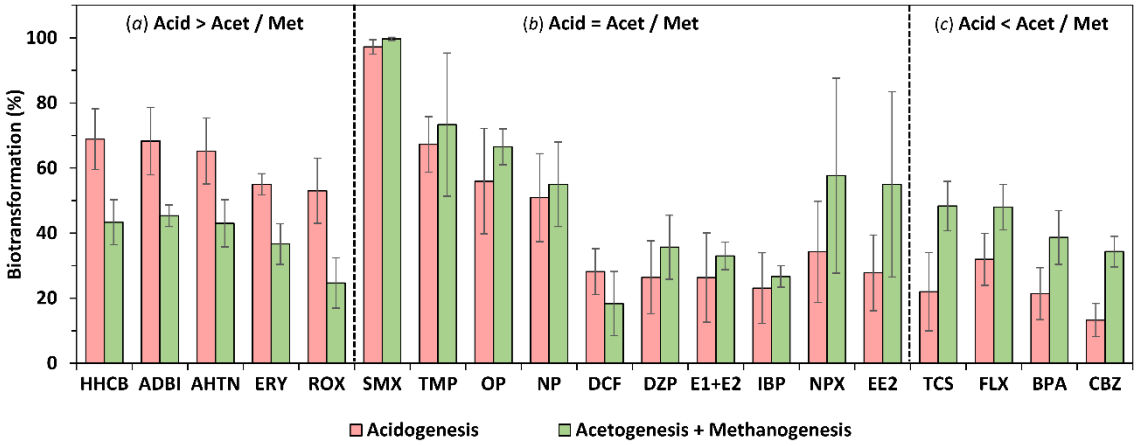


Fig. 6. Comparison between the biotransformation of OMPs during acidogenesis (n=15, fermentation reactor) and acetogenesis/methanogenesis (n=3, methanogenic reactor [36]). The compounds were divided into 3 groups: (a) Acid > Acet/Met, (c) Acid < Acet/Met (biotransformation differences ≥ 15 percentage points and $p \leq 0.05$); and Acid = Acet/Met (biotransformation differences < 15 percentage points and/or $p > 0.05$).

Finally, as previously pointed out in Fig. 4, the anaerobic biotransformation of some compounds (i.e., TMP and NPX) does not completely occur during acidogenesis, thus other anaerobic steps should be involved. However, according to the results of Gonzalez-Gil et al. [36] for TMP and NPX (Fig. 6), these differences can neither be explained by heteroacetogenesis and aceticlastic methanogenesis. Wolfson et al. [87] have recently found that NPX is possibly O-demethylated through a consortium of homoacetogens, acetate oxidizers, and hydrogenotrophic methanogens with a syntrophic relationship. Actually, the demethylation of the methoxy groups of NPX and TMP is quite common in anaerobic processes [15]. However, the activities of this microbial consortium were not promoted in the acidogenic and methanogenic reactors,

551 which could explain the higher biotransformation efficiencies (≥ 95%) achieved in the
552 overall anaerobic digestion (Fig. 4) for NPX and TMP in comparison with the results
553 attained in the acidogenic and methanogenic reactors (Fig. 6).

554

555 **4. CONCLUSIONS**

556 The main conclusion of this study is that the acidogenic phase of the anaerobic
557 digestion processes is key in the biotransformation of OMPs, while the hydrolysis of
558 carbohydrates does not significantly contribute to the cometabolic transformation of the
559 studied compounds. The biotransformation of HHCB, ADBI, AHTN, ERY and ROX
560 was favored under acidogenic conditions compared to acetogenesis/methanogenesis. On
561 the contrary, the biotransformation of other compounds, e.g. TCS, FLX, BPA, and CBZ
562 depends on acetogenesis/methanogenesis. Thus, the separation of the
563 hydrolytic/acidogenic and acetogenic/methanogenic phases in anaerobic treatment
564 technologies can be a promising alternative for enhancing OMPs biotransformation.

565 The molecular structure of OMPs appears to be an important factor in their anaerobic
566 biotransformation. Namely, the presence of EDGs might explain the high
567 biotransformation of some compounds (e.g., SMX and TMP), while EWGs could have
568 a negative effect (e.g., CBZ, IBP, DCF, FLX, TCS, DZP, and CTL). However, to
569 explain the differences found between the biotransformation during acidogenesis,
570 methanogenesis and the overall anaerobic digestion process, other aspects, such as the
571 specific enzymatic activities promoted should be considered.

572

573 **Disclosure statement**

574 There is no conflict of interest by the authors.

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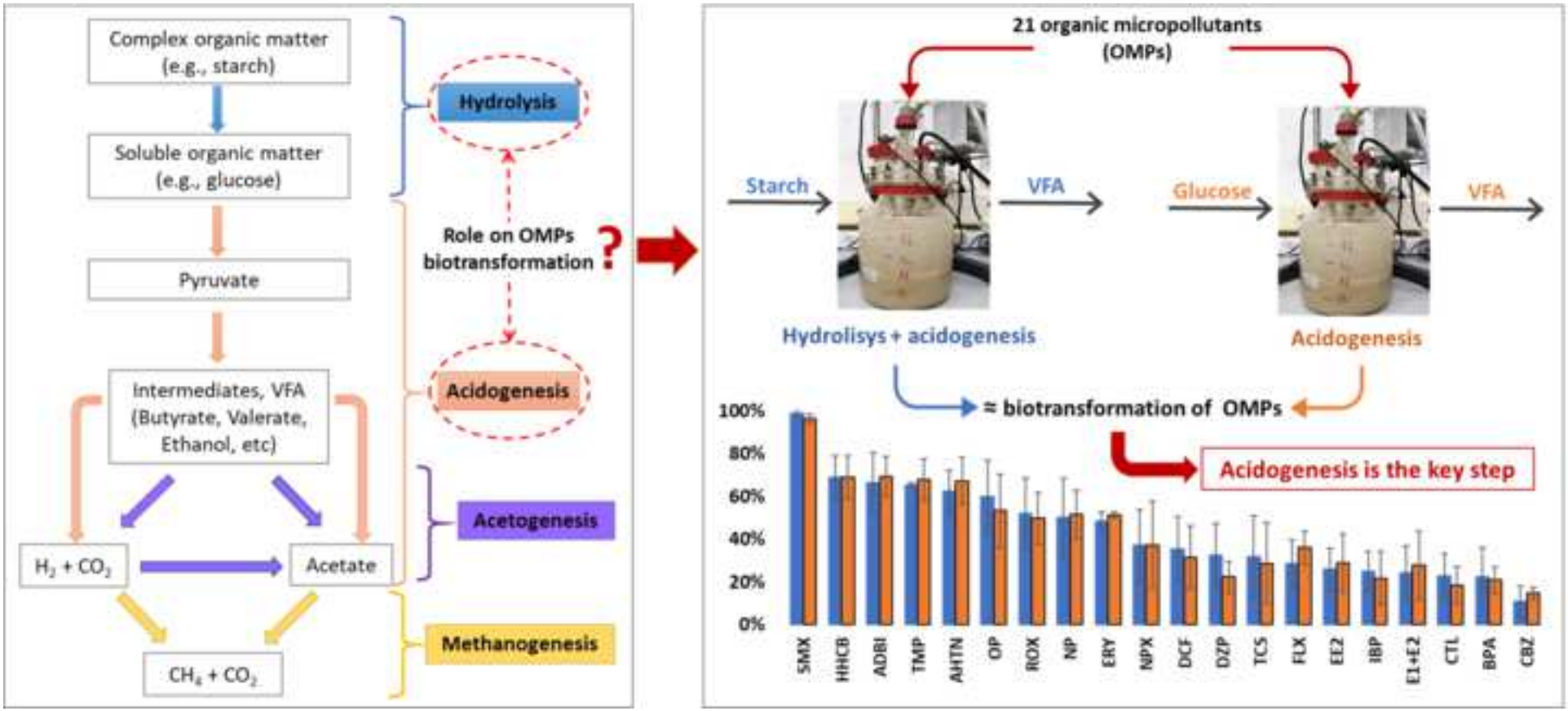
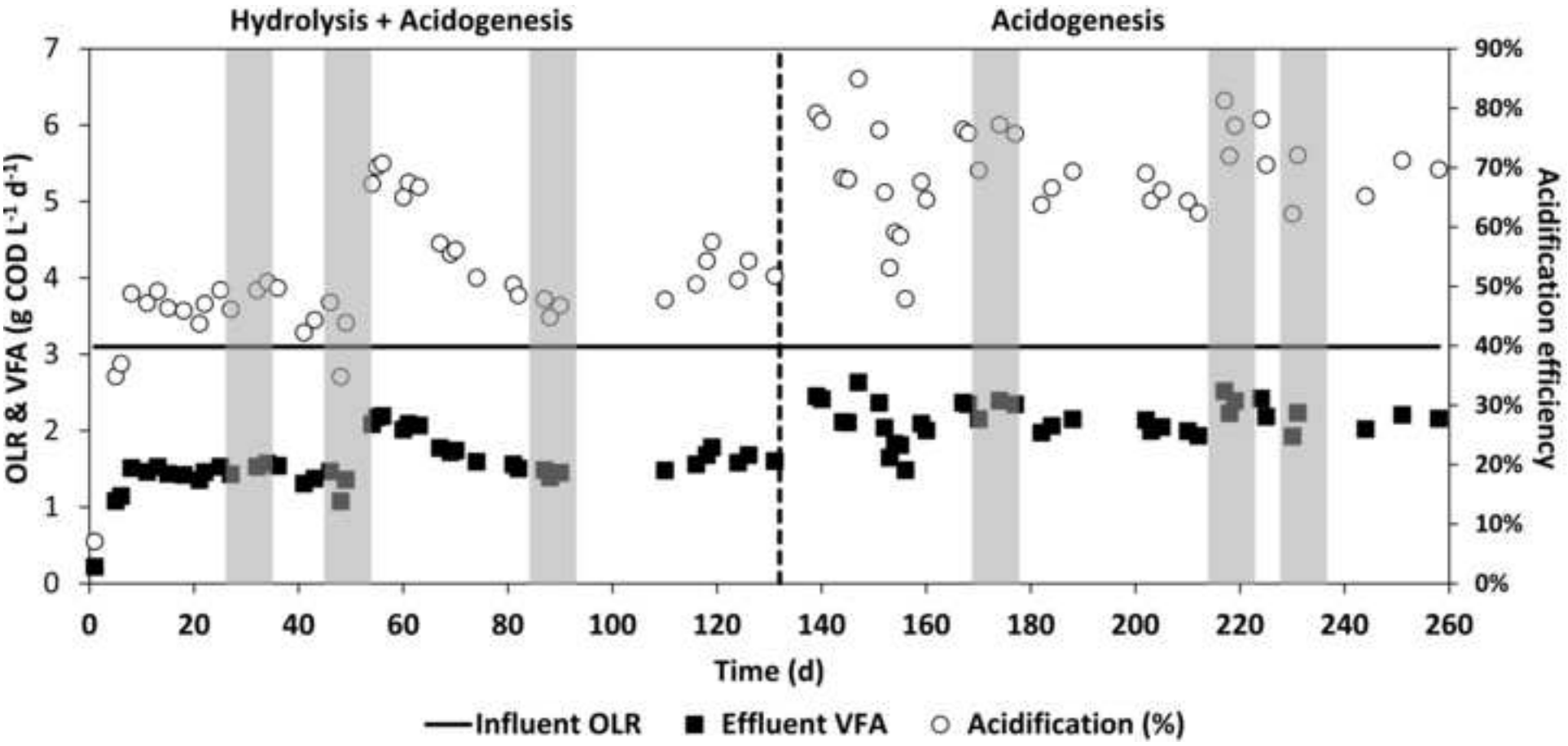
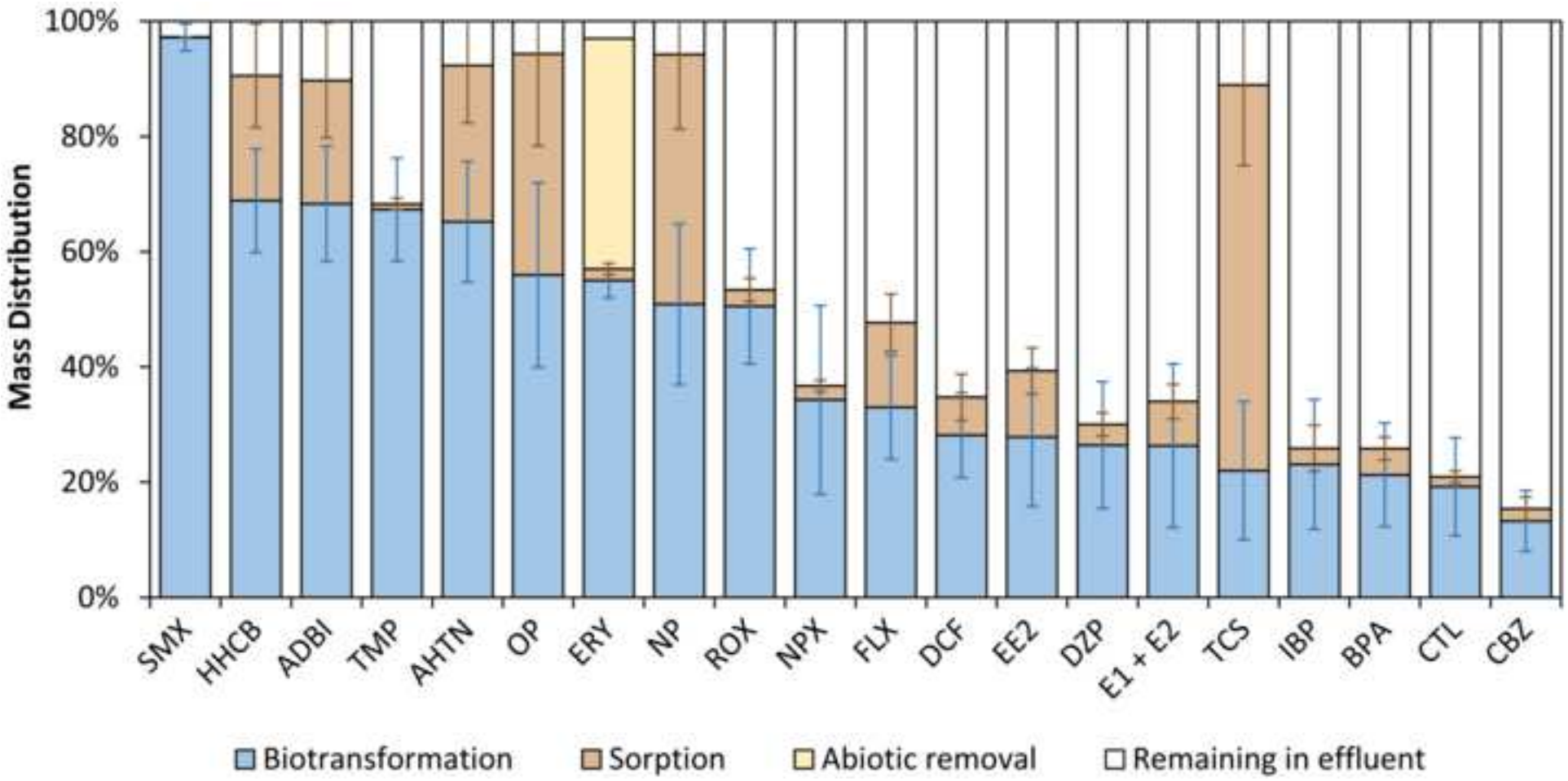
Graphical Abstract



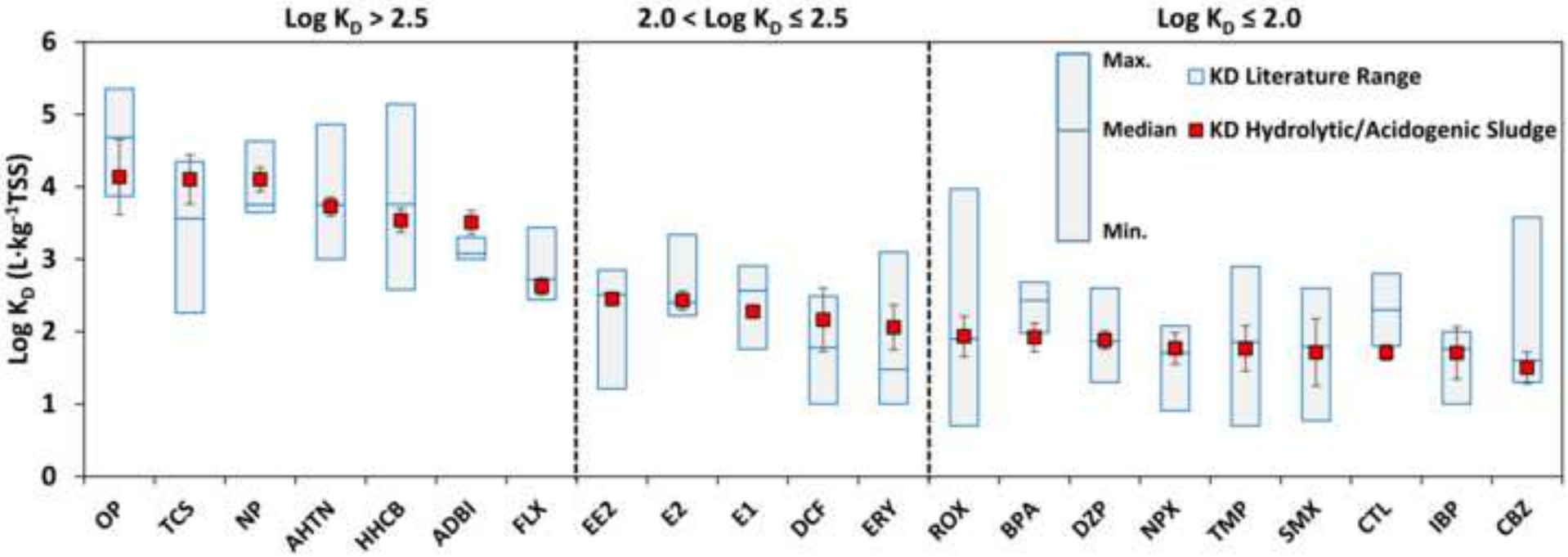
Figure


Hydrolysis + Acidogenesis
Acidogenesis
OLR & VFA (g COD L-1 d-1)
Acidification efficiency
Time (d)
Influent OLR
Effluent VFA
Acidification (%)

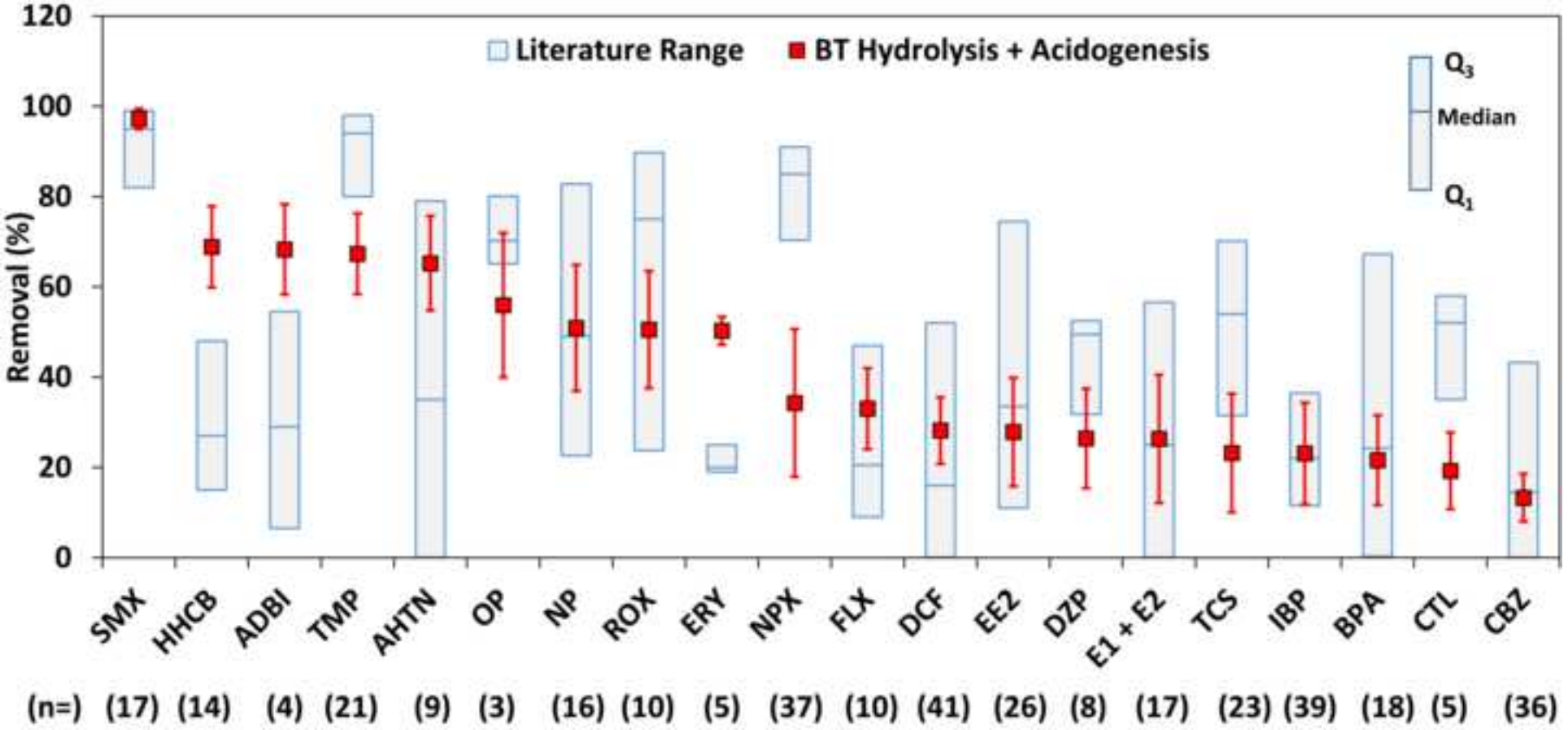
Figure


Mass Distribution
100%
80%
60%
40%
20%
0%
SMX
HHCB
ADBI
TMP
AHTN
OP
ERY
NP
ROX
NPX
FLX
DCF
EE2
DZP
E1 + E2
TCS
IBP
BPA
CTL
CBZ
Biotransformation
Sorption
Abiotic removal
Remaining in effluent

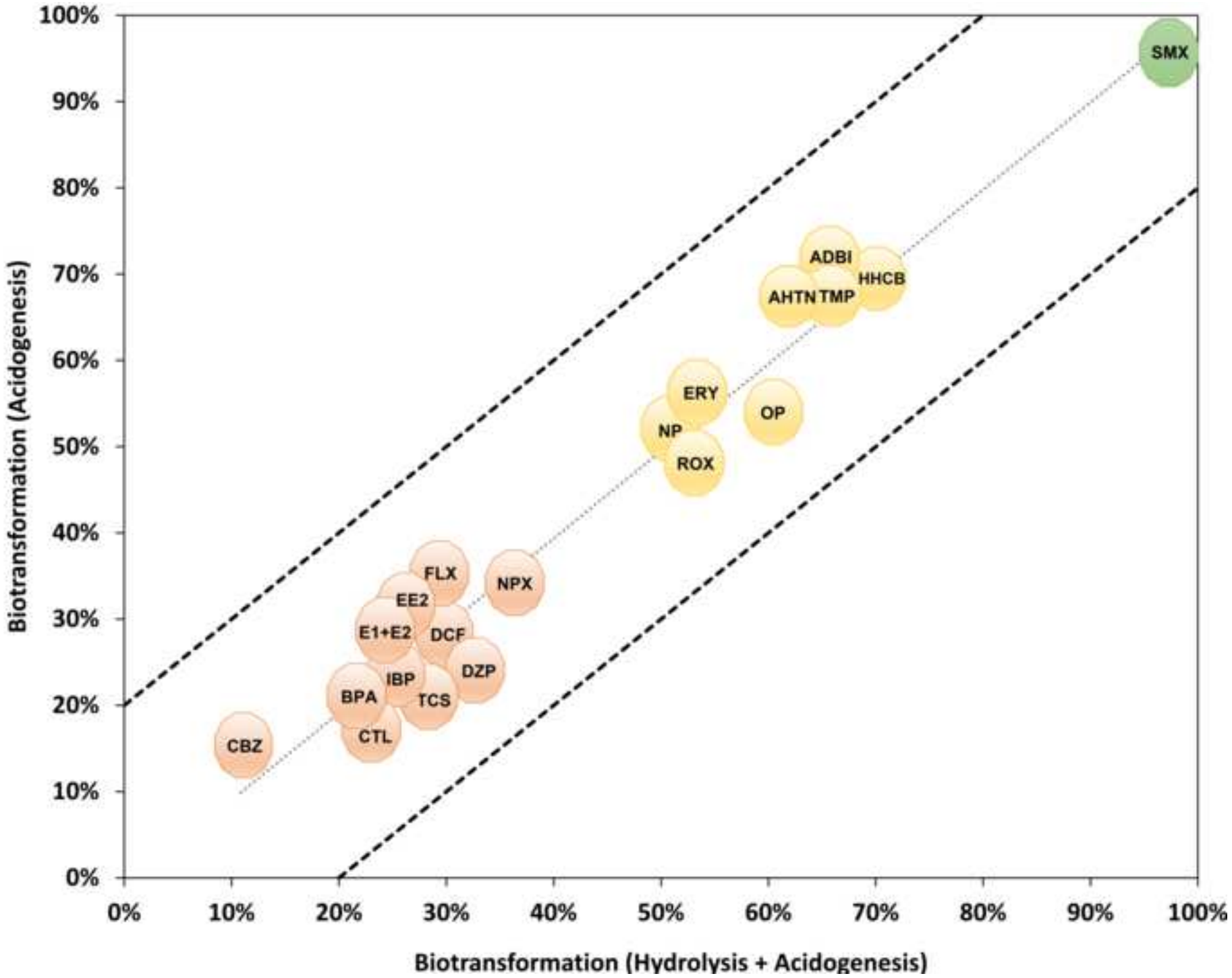
Figure


Log K_D > 2.5
2.0 < Log K_D ≤ 2.5
Log K_D ≤ 2.0
Log K_D (L·kg^{-1}TSS)
0
1
2
3
4
5
6
Max.
Median
Min.
KD Literature Range
KD Hydrolytic/Acidogenic Sludge
OP
TCS
NP
AHTN
HHCB
ADBI
FLX
EE2
E2
E1
DCF
ERY
ROX
BPA
DZP
NPX
TMP
SMX
CTL
IBP
CBZ

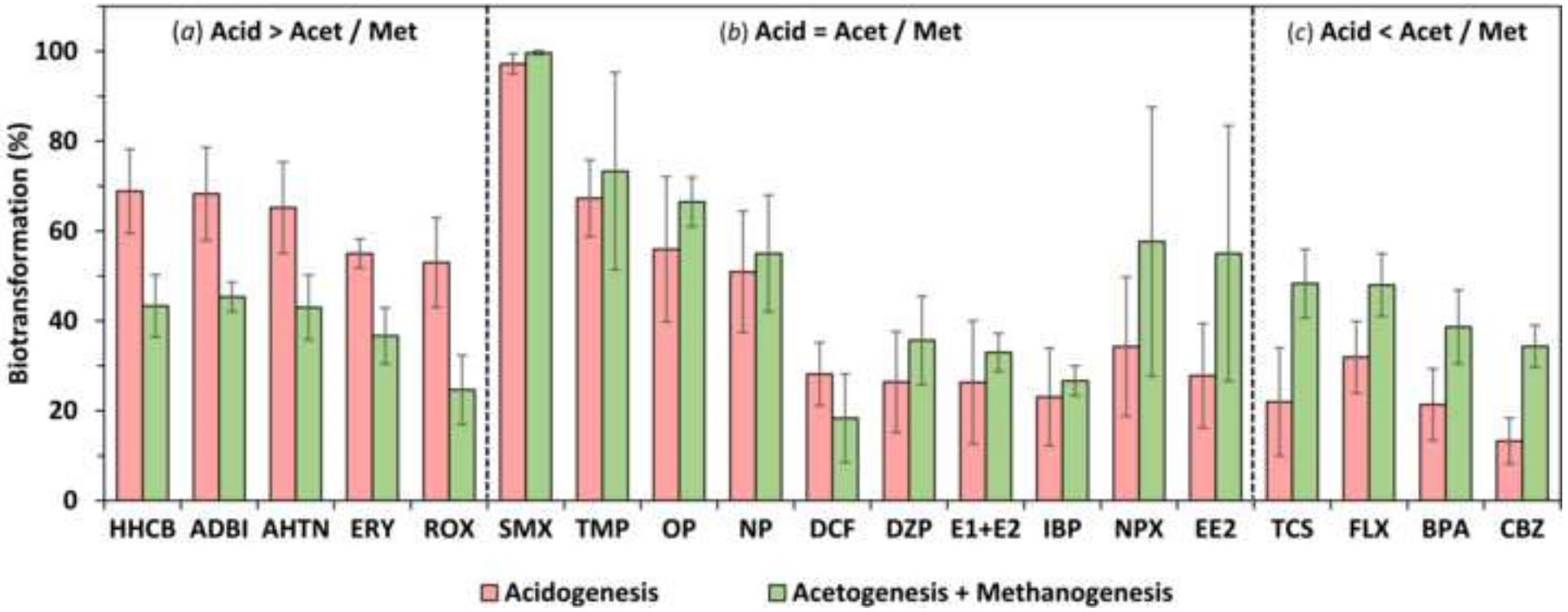
Figure


Literature Range
BT Hydrolysis + Acidogenesis
Q3
Median
Q1
Removal (%)
120
100
80
60
40
20
0
SMX
HHCB
ADBI
TMP
AHTN
OP
NP
ROX
ERY
NPX
FLX
DCF
EE2
DZP
E1 + E2
TCS
IBP
BPA
CTL
CBZ
(n=) (17) (14) (4) (21) (9) (3) (16) (10) (5) (37) (10) (41) (26) (8) (17) (23) (39) (18) (5) (36)

Figure


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Biotransformation (Acidogenesis)
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Biotransformation (Hydrolysis + Acidogenesis)
SMX
ADBI
HHCB
AHTN
TMP
ERY
OP
NP
ROX
FLX
NPX
EE2
E1+E2
DCF
DZP
IBP
BPA
TCS
CTL
CBZ

Figure


(a) Acid > Acet / Met
(b) Acid = Acet / Met
(c) Acid < Acet / Met
Biotransformation (%)
100
80
60
40
20
0
HHCB
ADBI
AHTN
ERY
ROX
SMX
TMP
OP
NP
DCF
DZP
E1+E2
IBP
NPX
EE2
TCS
FLX
BPA
CBZ
Acidogenesis
Acetogenesis + Methanogenesis